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- METHODS OF
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Dinitronaphthalene

Follow the procedure for dinitrobenzene with sodium hydroxide in acetone (page 12), but use a 5-gram sample and read at 410 mμ.

2,4-Dinitroanisole

The reaction of potassium cyanide with metadinitro compounds to give a red purpuric acid structure [40] is applicable.[41] Sulfur, as from rubber stoppers, will destroy the color. The color developed by shaking a metadinitro compound with concentrated sodium hydroxide is usually too sensitive for this determination but applicable to very small amounts. Unless 1-chloro-2,4-dinitrobenzene, which sometimes occurs as an impurity, is removed, it interferes.

Sample—*Insecticidal dust.* Weigh a sample expected to contain about 40 mg. of dinitroanisole. Stir with five successive portions of acetone, decanting through a Gooch crucible. Wash the filter until nearly to volume and dilute to 100 ml. The clear solution may be yellow because of pyrethrum oleoresins.

Procedure—To a 10-ml. aliquot add 5 ml. of 0.5 [illegible] cent potassium cyanide solution and mix. Read after one hour at [illegible] against an acetone blank.

Dinitrocresol

The blue color of dinitrocresol with sodium hydroxide in anhydrous acetone [42] has been discussed in more detail for dinitrobenzene (page 11) where the procedure is somewhat different. The method is designed to determine 0.001–0.1 per cent dinitrocresol formed in mononitrotoluene. Another form of the procedure determines 3,5-dinitro-*o*-cresol in urine.[43]

Procedure—*Mononitrotoluene.* Shake a 10-gram sample of mononitrotoluene with 75 ml. of 0.5 per cent sodium hydroxide solution for

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[40] V. Anger, *Mikrochim. Acta* **2**, 3-8 (1937).

[41] Milton S. Schechter and H. L. Haller, *Ind. Eng. Chem., Anal. Ed.* **16**, 325-6 (1944).

[42] F. L. English, *Anal. Chem.* **20**, 745-6 (1948); Cf. Walter Fischer, *Z. anal. Chem.* **112**, 91-6 (1938).

[43] V. H. Parker, *Analyst*, **74**, 646-7 (1949).

1 minute and dilute to 100 ml. with the hydroxide solution. After 5 minutes, filter and read at 410 mμ against the sodium hydroxide solution.

Urine. Add 5 ml. of methylethyl ketone to 5 ml. of urine, and follow with 2 grams of a 9:1 mixture of sodium chloride-sodium carbonate. Shake vigorously and centrifuge. Read the upper layer against methylethyl ketone at 430 mμ.

Trinitrobenzene

The color developed by trinitrobenzene in ethanol with sodium hydroxide is read in the presence of major amounts of dinitrobenzene.[44] In acetone, both give colors which darken with time. In ethanol, only the trinitro compound gives a color. Ammonia reacts similarly, but the color fades quickly. With large excess present, the amount of sodium hydroxide is not critical.

Sample—*Dinitrobenzene.* Dissolve 0.1 gram of dried powdered dinitrobenzene in ethanol and dilute to 50 ml. for the use of aliquots.

Procedure—Measure an aliquot containing 0.1–1 mg. of trinitrobenzene and add 0.5 ml. of 10 per cent sodium hydroxide solution. Dilute to 50 ml. with ethanol and read at 502 mμ within 10 minutes against a reagent blank.

Trinitrotoluene

The usual form of trinitrotoluene, designated as α or 2,4,6, is estimated in aqueous solution after treatment with sulfite and sodium hydroxide.[45] Sulfite alone gives less color and hydroxide a different one with a maximum at 460 mμ. The procedure is complicated by the effect of time in converting the trinitrotoluene to a colored complex which absorbs at a different wave length. Then the color must be read for each form of the test substance and the values combined. To complicate the determination further, the color derived from trinitrotoluene conforms to Beer's law, but that from the colored complex does not. The colored complex cannot be reduced to the original test substance. Ammonium picrate may interfere, but can be corrected for,

[44] M. L. Moss and M. G. Mellon, *Ind. Eng. Chem., Anal. Ed.* **14**, 861-2 (1942).

[45] C. C. Ruchhoft and William G. Meehler, *Ibid.* **17**, 430-4 (1945).

after determination by a modified procedure (page 24). The red-violet color with diethylaminoethanol is also read.[46]

In admixture with other nitrated toluenes, sodium bisulfite in acetone gives a specific color with α-trinitrotoluene.[47] Samples recovered from air are reduced with titanium trichloride, diazotized, and coupled with dimethyl-α-naphthylamine.[48]

The blue color of sodium hydroxide with dinitrobenzene or dinitrotoluene in anhydrous acetone is reddish when given by trinitrotoluene.[49] If both are present, the hues are distinctly different but not simple to separate photometrically.[50] The red color is stable for about 30 minutes, but if cyclohexanone is substituted for acetone, an intense red color results which is stable for about a week. A disadvantage is that alkali is only sparingly soluble in cyclohexanone and the two must be shaken thoroughly before there is any reaction between the test substance and the alkali. If the cyclohexanone is admixed with methylethyl ketone, the color produced is intense, permanent, and conforms to Beer's law. This reaction is also applied to tetryl [2,4,6-trinitrophenyl-1-methylnitramine] and differs only in hue when given by trinitrotoluene. It follows that, if both are present, they will interfere.

Semi-permanent tubes containing trinitrotoluene in butanol with 0.1 ml. of 0.56 per cent potassium hydroxide per 10 ml. are useful as rough standards for comparison against samples in butanol.[51]

Samples—*Air*. Bubble the sample through 10 ml. of isopropanol in a midget impinger (Vol. II, page 64) in the course of 20 minutes. For concentrations less than 0.1 mg. per cubic meter, an even longer period is necessary. Dilute to 10 ml. and develop color by reduction, diazotizing, and coupling with dimethyl-α-naphthylamine.

Water and sewage. Use as received or after appropriate dilution and develop with sulfite and hydroxide.

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[46] F. H. Goldman and D. E. Rushing, *J. Ind. Hyg. Toxicol.* **25**, 164-71 (1943).

[47] G. Halfter and H. Winkler, *Die Chemie* **57**, 124-5 (1944).

[48] Sherman S. Pinto and John P. Fahy, *J. Ind. Hyg. Toxicol.* **24**, 24-6 (1942); Sherman S. Pinto and William L. Wilson, *Ibid.* **25**, 381-90 (1943).

[49] K. Kay, *Canadian J. Res.* **19B**, 86 (1941); W. M. Cumming and W. G. D. Wright, *Brit. J. Ind. Med.* **2**, 83-5 (1945); I. M. Korenman and A. M. Fisher, *Zavodskaya Lab.* **14**, 1058-60 (1948).

[50] M. L. Moss and M. G. Mellon, *Ind. Eng. Chem., Anal. Ed.* **14**, 861-2 (1942).

[51] Thomas E. Cone, Jr., *U. S. Naval Med. Bull.* **41**, 219-20 (1943).

Hexanitrodiphenylamine present.[52] Mix 100 ml. of aqueous solution with 20 grams of sodium chloride and 5 ml. of 1:2 ammonium hydroxide. Extract with 10, 10, and 10 ml. of chloroform. Save the aqueous layer for recovery of hexanitrodiphenylamine. Dry the combined extracts with anhydrous sodium sulfate and evaporate to dryness. Dissolve in 20 ml. of anhydrous acetone and develop with sodium hydroxide in anhydrous acetone.

Urine. Dilute a suitable aliquot of a 24-hour sample to 7.5 ml. and use for diazotizing and coupling with N-(1-naphthyl)ethylenediamine dihydrochloride.

Tissue. Extract with trichloracetic acid of suitable concentration, diazotize the filtrate, and couple with N-(1-naphthyl)ethylenediamine dihydrochloride.

Alternatively prepare acetone filtrates from 1 part of tissue and 3 parts of absolute acetone. Evaporate the acetone at room temperature, suspend in 1 per cent bicarbonate solution, and extract with ether. Determine by reduction, diazotizing, and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride.

Procedure—*By sulfite and hydroxide. Uncolored solutions.* In a 50-ml. sample estimated to contain 0.02-1 mg. of trinitrotoluene dissolve approximately 1 gram of anhydrous sodium sulfite. After 5 minutes add 1 ml. of 8 per cent sodium hydroxide solution and mix. Filter after 10 minutes to remove precipitated calcium salts, etc., and dilute to a known volume with fresh 0.02 per cent sodium hydroxide-0.06 per cent sodium sulfite solution. This will fall in the range 1:4-1:50. Dilution with water would lessen the color. Read at once at 505 mμ and every minute against a distilled water blank until a maximum is reached which will be within 10 minutes.

Colored solutions. Treat a sample as though uncolored. Also dilute a filtered sample with 0.03 per cent sodium carbonate solution and read at once at 460 mμ and 505 mμ. Read the sample diluted with sodium hydroxide-sulfite against a blank of the same degree of aqueous dilution as the original sample.

For a 1:50 dilution of the colored complex the ppm. is (E/0.29) the dilution factor. At 1:5–1:25 dilution the factor is 0.26. An instrumental correction may also be required. For the uncomplexed trinitrotoluene, calculate as usual from the value with the diluted sample as blank.

[52] F. Seifert, *Vom Wasser*, 17, 89-92 (1949).

By sodium hydroxide in anhydrous acetone. Measure out an aliquot of sample in anhydrous acetone containing 0.05-0.2 mg. of trinitrotoluene. Dilute to about 5 ml. with anhydrous acetone and add 1.5 ml. of 50 per cent sodium hydroxide solution. Mix well, dilute to 10 ml. with anhydrous acetone, and mix again. Filter and, after about 7 minutes, read at 540 mμ against a reagent blank.

By reduction and coupling with dimethyl-α-naphthylamine. As reducing reagent, dissolve 10 ml. of 20 per cent titanous chloride solution in 10 ml. of concentrated hydrochloric acid and dilute with water to 100 ml. Also dissolve 5 grams of ferric alum in water, add 5 ml. of 1:5 sulfuric acid, and dilute to 100 ml. By mixing 1 ml. of the ferric reagent with 0.5 ml. of the titanium solution, 2 drops of 10 per cent potassium thiocyanate should show the characteristic ferric thiocyanate color.

To a 5-ml. aliquot of sample in isopropanol containing not over 0.2 mg. of trinitrotoluene, add 5 ml. of 1:10 sulfuric acid and 0.5 ml. of titanous chloride solution. Heat for 10 minutes in a water bath, remove, and add 1 ml. of the ferric alum reagent to destroy excess titanous chloride. Cool to room temperature and add 1 ml. of 0.1 per cent sodium nitrite solution. Mix and within 15 seconds add 1 ml. of 0.5 per cent ammonium sulfamate solution. Mix and add within the next 15 seconds 1 ml. of 1 per cent dimethyl-alpha-naphthylamine in ethanol. After 1 hour, dilute to 15 ml. and read at 530 mμ.

With methylethyl ketone in cyclohexanone. See the procedure under tetryl (page 24).

Trinitrophenol, Picric Acid

Picric acid as ammonium picrate is determined spectrophotometrically.[53] Complications arise when trinitrotoluene is also present. The color in alkaline solution is intensified if necessary by addition of a know amount of glucose.[54]

Samples—*Water and sewage.* Filter or centrifuge to remove turbidity. Under extreme conditions coagulate with alum, recognizing that results at the 10 ppm. level may be 25 per cent low. Natural hardness is without effect. Dilute to under 40 ppm.

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[53] C. C. Ruchhoft and Francis I. Norris, *Ind. Eng. Chem., Anal. Ed.* **18**, 480-3 (1946).

[54] R. Stöhr and F. Scheibl, *Mikrochemie ver. Mikrochim. Acta* **36/37**, 362-5 (1951).

Procedure—*Trinitrotoluene absent.* Read the ammonium picrate solution at 460 mμ or in the ultraviolet at 335 mμ.

Trinitrotoluene possibly present. Read at 460 mμ and 505 mμ, after dilution if necessary with 0.03 per cent sodium carbonate. If the extinction at 460 mμ is only slightly higher than at 505 mμ, colored trinitrotoluene and possibly organic color are indicated rather than ammonium picrate. If the 460 mμ value is substantially higher than that at 505 mμ, and the latter is significant, both ammonium picrate and colored trinitrotoluene are probably present. A value at 460 mμ, but none at 505 mμ, indicates only ammonium picrate.

If trinitrotoluene is present, carry through the technic for it in colored solution (page 22). A gain in extinction at 505 mμ indicates only that the two forms of trinitrotoluene are present. If, after development, the value at 505 mμ increases but is lower than the 460 mμ value, both ammonium picrate and trinitrotoluene are present. If both values are the same or lower after treatment, ammonium picrate is absent.

If both may be present, mix 50 ml. of diluted sulfite-hydroxide treated sample with 5 ml. of concentrated hydrochloric acid. Read after 1 minute at 460 mμ and 505 mμ. Decolorization indicates both are absent. A considerable decrease from 460 mμ which is ammonium picrate to 505 mμ where ammonium picrate shows no absorption in the concentration of sample used, indicates ammonium picrate. This 460 mμ value gives the ammonium picrate by direct calculation. The appropriate correction can then be applied to the trinitrotoluene values if necessary.

2,4,6-Trinitrophenyl-1-methylnitramine, Tetryl

The color reaction with alkali in anhydrous acetone given by dinitrobenzene and trinitrotoluene is also given by tetryl. Stability is increased and other advantages are introduced by use of a less volatile solvent. The color is a brownish-red with tetryl and of lesser stability than with trinitrotoluene.[55] Suitable atmosphere analysis apparatus is the midget impinger (Vol. II, page 64).

Procedure [56]—As reagent, mix equal volumes of cyclohexanone and methylethyl ketone and store in a dark bottle. Place 10 ml. of this reagent in the sampling tube of the midget impinger, attach to the pump,

[55] W. M. Cumming and W. G. D. Wright, *Brit. J. Ind. Med.* 2, 83-5 (1945).
[56] The identical procedure is applicable to trinitrotoluene.

and bubble in the atmosphere. Operate at about 1 stroke per 5 seconds. Remove the tube, shake with 0.5 ml. of 12 per cent potassium hydroxide solution until a maximum color is attained, and allow suspended alkali to settle out for a minute. Read the color at an appropriate wave length.

Hexanitrodiphenylamine, Dipicrylamine

The solution from which trinitrotoluene has been separated is used for estimation of hexanitrodiphenylamine.

Procedure—Extract the sample (page 22) with 10, 10, and 10 ml. of ether. Extract the combined ether extracts with 10, 10, and 10 ml. of saturated aqueous sodium chloride solution. Dry the ether extracts with anhydrous sodium sulfate and filter. Dry and bake at 180° for an hour. Cool and take up in about 90 ml. of 1:100 ammonium hydroxide solution. Dilute to 100 ml. and read against a reagent blank.

O-O-Diethyl-O-*p*-Nitrophenyl Thiophosphate, Parathion

The strongly chromophoric *p*-nitrophenyl group in parathion in ethanol causes a broad and intense absorption band in the near ultraviolet with a maximum near 274 mμ.[57] It is also estimated by reduction to the amine, diazotizing, and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride.[58] Aniline and some of its homologues give substantially the same color effect.[59] Methyl anthranilate will interfere unless the solution is washed three times with 1:2.5 hydrochloric acid.

Both parathion and dimethyl parathion are conveniently determined by saponification to *p*-nitrophenol with 4 per cent ethanolic sodium hydroxide.[60] Free *p*-nitrophenol in the product introduces complications, necessarily overcome. Other impurities are *O*-ethyl-*O,O*-bis(*p*-nitrophenyl)thiophosphate, *p*-nitrophenetol, and triethylthiophosphate. The direct color of the *p*-nitrophenol is measured. The monoethylthiophosphate is ordinarily present only to a minor extent. The *p*-nitrophenetol produces only traces of *p*-nitrophenol during hydrolysis and triethylthiophosphate does not interfere. If *O,O*-diethyl-*O,o*-nitrophenyl thiophosphate is present it is determined by the absorption at

[57] Robert C. Hirt and J. B. Gisclard, *Anal. Chem.* **23**, 185-7 (1951).

[58] P. R. Averell and M. V. Norris, *Ibid.* **20**, 753-6 (1948); J. C. Gage, *Analyst* **75**, 189 (1950).

[59] F. I. Edwards, Jr., *Anal. Chem.* **21**, 1415-6 (1949).

[60] J. A. A. Ketelaar and J. E. Hellingman, *Ibid.* **23**, 646-50 (1951).

450 mμ where it is equal to *p*-nitrophenol and at 405 mμ where it is only one-fourth as great.

Sample—*Benzene extracts.* Pass through a sorption column consisting of 1 part of Hyflo-Supercel to 2 parts of Atapulgus clay. This removes interfering pigments. Determine by N-(1-naphthyl)-ethylene diamine dihydrochloride.

Citrus peel. Transfer a 5-gram sample of less than 20-mesh material to a Babcock cream bottle. Add benzene to wet the sample but not provide excess, usually 5–6 ml. Stopper overnight. Add water to about 75 per cent of the volume and agitate mildly to hydrate the pulp. After a few minutes dilute to volume and centrifuge to separate the benzene. Decant that layer and recentrifuge. Use an aliquot for determination by N-(1-naphthy)ethylenediamine dihydrochloride.

Citrus oil. Use as is.

Liquid or technical parathion. Add a sample equivalent to 0.05–0.1 gram of parathion to a few ml. of ethanol. Reflux with 10 ml. of fresh 4 per cent ethanolic sodium hydroxide for 15 minutes. Cool and dilute to 100 ml. with ethanol plus 50 ml. of water. This sample solution is in 50 per cent ethanol containing about 0.4 per cent of sodium hydroxide. Determine as *p*-nitrophenol.

Wettable powders. Extract a sample containing 0.05–0.1 gram of parathion with ether. Evaporate the ether extracts to dryness. Take up the residue in a few ml. of ethanol and continue as for parathion from "Reflux with 10 ml. of . . . ."

Procedure—*Air by ultraviolet absorption.* Absorb the contaminant from an appropriate volume of air in ethanol. Dilute with the same solvent, if necessary, and read at 274 mμ.

By N-(1-naphthyl)ethylenediamine. General. Dilute an aliquot of sample in benzene to 10 ml. with benzene. Evaporate to dryness in a stream of air and take up in 10 ml. of ethanol. Add 10 ml. of water and 2 ml. of 1:1 hydrochloric acid. Mix and add 0.2 gram of zinc dust. Cover and boil gently for 5 minutes. Filter and dilute to about 40 ml. Add 1 ml. of 0.25 per cent sodium nitrite solution and mix. After 10 minutes add 1 ml. of 2.5 per cent ammonium sulfamate solution and mix. After another 10 minutes add 2 ml. of 1 per cent aqueous N-(1-naphthyl)ethylenediamine dihydrochloride and dilute to 50 ml. After 10 minutes read at 550 mμ against a reagent blank.

Citrus oil and extracts. Prepare an acid-alcohol solution of 60 ml. of 99 per cent isopropanol and 5 ml. of concentrated hydrochloric acid and dilute to 100 ml. with water. Mix 0.4 gram of zinc dust, 0.6 gram of paraffin, and 4 ml. of this acid-alcohol solution. Add 0.2 ml. of citrus oil or 1 ml. of the benzene extract of citrus origin. Bring to boiling within 2 minutes and boil gently for exactly 10 minutes. Cool in water and filter through cotton. The paraffin will have solidified and retain the excess of zinc. Rewarm this with 1 ml. of acid-alcohol and cool. Filter and wash with acid-alcohol to total about 9 ml. Add 4 drops of 0.25 per cent sodium nitrite solution, mix, and let stand for 10 minutes. Add 4 drops of 2.5 per cent ammonium sulfamate solution, mix, and let stand for 3 minutes. Dilute to 10 ml. with acid-alcohol and remove a 5-ml. aliquot. The residue is a sample blank. At the end of 3 minutes add 4 drops of 0.1 per cent aqueous N-(1-naphthyl)ethylenediamine dihydrochloride. After 10 minutes dilute to 10 ml. with acid-alcohol, filter, and read at 555 mμ against the sample blank similarly diluted.

As p-nitrophenol. Dilute the sample as *p*-nitrophenol in alkaline 50 per cent ethanol to 0.3 mg. per 100 ml. with 1 per cent sodium hydroxide in 50 per cent ethanol. Deviations of ± 10 per cent in ethanol or alkali do not affect the color. Read at 405 mμ. Corrections for impurities must be applied.

Dimethylparathion

Dimethylparathion is determined as *p*-nitrophenol by the method described for parathion.

Sample—Dissolve a sample equivalent to 0.05–0.1 gram in a few ml. of ethanol and proceed as for liquid or technical parathion (page 26) from "Reflux with 10 ml. . . . ."

2-Nitro-1,1-bis(*p*-chlorophenyl)alkanes, Dilan

Dilan is a mixture of 2 parts of the butane derivative and 1 part of the propane compound. By shifting the equilibrium to the aci form with alkali a complex is formed with ferric ion.[61]

Samples—*Plant or animal.* Pulp a sample containing 0.1–2 mg. of Dilan in water, adding a little hexane if desired. Extract with 250 ml.

[61] Laurence R. Jones and John A. Riddick, *Ibid.* 23, 349-51 (1951).

of hexane in a Soxhlet for 1 hour. Evaporate the extract, take up with ethanol, and dilute to 7 ml. with the same solvent.

Procedure—*Fat-free.* To the 7-ml. sample in ethanol at once add 1 ml. of 2 per cent sodium hydroxide in methanol. Mix and after 10 minutes add 3 ml. of 0.5 per cent ferric chloride in 0.3 *N* hydrochloric acid in 50 per cent methanol. Dilute to 12.5 ml. with ethanol and read at 490 mμ against a reagent blank.

Fat present. If a turbid solution results due to fat, add 5 ml. of ether after the ferric chloride reagent. Then dilute to 25 ml. with ethanol and read at 490 mμ against a reagent blank.

CHAPTER 2

ALIPHATIC AMINES AND AMIDES[1]

THE classification is such that most primary, secondary, and tertiary amines fall in this chapter unless the primary use is such as to lead to classification by some other functional group. It follows that many aromatic compounds come into this classification when the amine is on a side chain. Some miscellaneous amine derivatives are added. Methods for hydrazine and hydroxylamine, which are usually considered as inorganic, are included. Chemically they are amines or closely related to them.

The general reactions are covered in part as relating to amino acids, a classification of primary amines following this one. Aside from that they are diazotizable and develop colors on coupling. Many develop stable colors with ferric salts. Others are determined by the color with dimethylaminobenzaldehyde.

ALIPHATIC AMINES

The general reaction of aliphatic amines with sodium 1,2-naptho-quinone-4-sulfonate is applicable to a large number in the absence of others.[2] Applied to amino acids in blood and urine [3] it determines these as a group with a low degree of accuracy.[4] Table 2 shows amines to which the reaction has been applied, including, as a matter of information, many to which the reaction is not satisfactorily applicable.

The intense color of amine picrates in solvent is also suitable for general estimation of aliphatic amines.[5] The reaction has been applied to biological samples [6] and to long-chain amines such as dioctylmethyl-

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[1] See Chapter 1, Volume III, for details of organization, condensation, etc.

[2] E. G. Schmidt, *Ind. Eng. Chem., Anal. Ed.* **11**, 99-100 (1939).

[3] Otto Folin, *J. Biol. Chem.* 377-91, 393-4 (1922); Otto Folin and Hilding Berglund, *Ibid.* **51**, 395-418 (1922).

[4] N. Howell Furman, George H. Morrison, and Arthur F. Wagner, *Anal. Chem.* 22, 1561-2 (1950).

[5] For more details see determination of trimethylamine by this method, page 40.

[6] Derek Richter, *Biochem. J.* 32, 1763-9 (1938); Derek Richter, Margaret H. Lee, and Denis Hill, *Ibid.* **35**, 1225-30 (1941).

amine.[7] It follows that more than one present will interfere with others. Thus the reaction determines ephedrine, benzedrine, β-phenylethylamine, mescaline, β-phenylisopropylmethylamine, and urotropin.[8] Often differential solubilities can be used for separation.

Table 2. Absorbance of Solutions of 2,4-Dinitrophenyl Derivatives of Amines

Amine	*Absorbance*	*Absorption Maximum mμ*
Ethanolamine	0.409	350
Isopropylamine	0.520	330
n-Hexylamine	0.507	330
Aniline	0.361	335
Benzylamine	0.420	325–330
Dodecylamine	0.326	330
Diethylamine	0.584	355
Di-*n*-propylamine	0.525	355
Di-*n*-butylamine	0.402	355–360
Piperidine	0.423	355
2,3-Dimethylpiperidine	0.364	360
Benzyl-*N*-ethylamine	0.417	350
Benzyl-*N*-*n*-butylamine	0.339	350–355
Dibenzylamine	0.193	340–345
d-Desoxyephedrine	0.507	355
N-Methylaniline	0.066	355–360
Diisopropylamine	0.000	...
Dicyclohexylamine	0.000	...

A solution of 1-fluoro-2,4-dinitrobenzene is applicable as a reagent with many primary and secondary aliphatic amines.[9] The absorbancies of some are given in Table 2 as measured at 3.5×10^{-5} M. The basic reaction is to use excess of the reagent, convert the excess to 2,4-dinitrophenol, separate 2,4-dinitrophenylamine from the 2,4-dinitrophenol, and read the amine. The reaction occurs readily, even with aniline.

Sample—*Solutions.* Neutralize the sample solution to just pink with phenolphthalein and dilute to a concentration of amine or amines being determined of 0.008–0.025 mg. per ml. Develop by 1,2-naphthaquinone-4-sulfonate.

Blood. To a 10-ml. sample add 1 gram of potassium carbonate. Add a drop of 0.1 per cent solution of tyramine to destroy traces of

[7] R. M. C. Hopewell and J. E. Page, *Analyst* 70, 17 (1945).

[8] C. Rizzoli, *Boll. soc. ital. biol. sper.* 25, 433-8 (1949).

[9] F. Sanger, *Biochem. J.* 39, 507-15 (1945); Floyd C. McIntyre, Lois M. Clements and Muriel Sproull, *Anal. Chem.* 25, 1757-8 (1953).

aldehydes. Add 5 ml. of petroleum ether and shake for 30 seconds to remove the amines, the tyramine remaining behind. Discard the aqueous layer and similarly extract the amines from the petroleum ether with 5 ml. of 1:35 sulfuric acid saturated with sodium bromide. Discard the petroleum-ether layer and neutralize the acid extract by dropwise addition of 50 per cent potassium hydroxide solution. Extract the amines from the prepared and neutralized solution with 3 ml. of petroleum ether and separate the extract for development by picric acid.

Procedure—*By 1,2-naphthoquinone-4-sulfonate.* Add the volume of 0.1 saturated sodium carbonate indicated in Table 3 according to the amine being determined and dilute to 10 ml. Add 2 ml. of fresh 0.5 per cent solution of 1,2-naphthoquinone-4-sulfonate and store in the dark for 24 hours. Add 2 ml. of a buffer containing equal volumes of 1:1 acetic acid and 5 per cent sodium acetate. Mix and add 2 ml. of 4 per cent sodium thiosulfate solution. Dilute to 25 ml. and read. In the case of mixed amines or those of unknown structure, it is convenient to use a dilution of a 0.0534 per cent solution of glycine in 1:120 hydrochloric acid containing 0.2 per cent sodium benzoate. Each ml. is equivalent to 0.1 mg. of amide nitrogen. As indicated in Table 3 the color match of some amines with a glycine standard is only fair.

By picric acid. To 3 ml. of sample containing 0.0005–0.005 mg. of test substance add 0.1 ml. of 2 per cent solution of picric acid in chloroform and dilute to 5 ml. with chloroform. Read after 12 hours at 445 mμ against a reagent blank.

By 1-fluoro-2,4-dinitrobenzene. To 0.1 ml. of sample containing 0.01–0.1 mg. of amine add 0.05 ml. of a solution containing 1.2 ml. of 1-fluoro-2,4-dinitrobenzene per 100 ml. of ethanol. Add 0.1 ml. of 0.84 per cent solution of sodium bicarbonate, mix, and heat at 60° for 20 minutes. Add 0.4 ml. of 0.8 per cent sodium hydroxide solution in 60 per cent dioxane and continue at 60° for 60 minutes. Dilute to 10 ml. and add 10 ml. of cyclohexane, or, in the case of highly water-soluble amines, use *s*-tetrachloroethane. Shake and read the organic solvent extract at the appropriate wave length as shown in Table 3 against a solvent blank.

Amides

The reaction of a concentrated solution of an amide with hydroxylamine hydrochloride gives an almost quantitative yield of an acethy-

TABLE 3. QUANTITATIVE NATURE OF THE REACTION BETWEEN VARIOUS AMINES AND SODIUM 1,2-NAPHTHOQUINONE-4-SULFONATE

Compound Studied	*0.1 Saturated Sodium (ml.)*	*Recovery from 0.7 mg. of Nitrogen in 5-ml. Sample with Glycine Standard (%)*	*Color Match with Glycine*
Allylamine	0.5	100.0	Good
o-Aminophenol	0.5	125.0	Fair, more orange
m-Aminophenol	0.4	154.0	Fair, more orange
p-Aminophenol	0.6	200.0	Fair, more orange
Ammonium hydroxide	0.4	95.9	Poor, too red
sec-Butylamine	0.6	50.5	Fair, too yellow
Isobutylamine	0.4	60.6	Poor, too yellow
Cadaverine dihydrochloride	0.4	99.0	Fair
Di-*n*-amylamine	0.6	102.0	Fair, too orange
Di-*n*-butylamine	0.7	101.1	Fair, too orange
Diisobutylamine	1.0	52.0	Good
Diethanolamine	0.3	83.3	Good
Diethylamine	0.7	97.6	Fair
Di-*n*-propylamine	0.8	106.1	Fair, too orange
Diisopropylamine		No reaction	
Ethanolamine	0.6	102.9	Good
Ethylamine	0.2	50.1	Good
Ethylenediamine		No reaction	
d-Glucosamine hydrochloride	0.1	96.4	Good
Methylamine	0.2	100.0	Perfect
Propanolamine	0.2	137.0	Fair, more orange
n-Propylamine	0.2	62.6	Fair, too yellow
Isopropylamine	0.1	47.6	Poor, too yellow
Putrescine dihydrochloride	0.5	102.0	Good
p-Sulfanilic acid	0.0	192.3	Fair, more orange
o-Toluidine hydrochloride	1.0	103.1	Good
Tyramine hydrochloride	0.5	111.0	Fair, more red

droxamic acid.[10] This has been developed for determination of a series of amides shown in Table 4.[11] The reaction is analogous to the conversion of esters or anhydrides into hydroxamic acids. The end product is developed with ferric chloride. The color fades slowly. Optimum conditions differ with different compounds as shown in the table. After the optimum time gradual decomposition of the product occurs. Barbiturates vary with the substituent. Betaines do not react.

Procedure—The sample should be in aqueous solution, of a percentage concentration approximating 0.01 *M*. Prepare the reagent by mixing equal parts of 16.4 per cent hydroxylamine sulfate solution

[10] C. Hoffmann, *Ber.* **22**, 2854 (1889).

[11] Felix Bergmann, *Anal. Chem.* **24**, 1367-9 (1952)

and 14 per cent sodium hydroxide solution. Add 2 ml. of this to no more than 1 ml. of sample, and if less than 1 ml. of sample is used dilute the whole to 3 ml. Hold at the appropriate temperature for the time specified in Table 4. After formation of the hydroxamic acid derivative, cool to room temperature and add 1 ml. of 1:3 hydrochloric acid and 1 ml. of 20 per cent ferric chloride hexahydrate solution in 1:160 hydrochloric acid. Read within 5 minutes at 540 mμ except for fluoroacetamide for which use 500 mμ.

TABLE 4. OPTIMAL CONDITIONS FOR CONVERSION OF AMIDES INTO HYDROXAMIC ACIDS

Name of Compound	*Temperature* (° C.)	*Reaction Time,* (Min.)	*Reading of 500-570 mμ Filter in Units per Micromole*
Acetamide	60	120	90
	26	480	103
N-Methylacetamide	60	420	57
Acetanilide	60	180	70
N^4-Acetylsulfanilamide	60	240	70
Acetylglycine	60	240	35
Fluoracetamide	26	60	62
Formamide	26	60	80
	60	10	75
Dimethylformamide	26	240	45
Succinimide	60	120	85
Caprolactam	60	420	41
Asparagine	60	180	38
Glutamine	60	180	35
Glutathione	60	120	48
Glycylglycine	60	120	25
Nicotinamide	26	480	45
N^1-Methylnicotinamide methosulfate (I)	26	360	45
Nicotinic acid methylamide	60	240	30
Coramine (nicotinic acid diethylamide)	60	480	6
Pantothenic acid, calcium salt	26	300	89
Barbitone	100	45	1.7
Pentobarbitone	60	300	1.5
Phenobarbitone	100	120	7.5
Evipan, sodium	100	30	9

LIPID AMINE-ALDEHYDES

The brown color developed by dried eggs in storage is an ether-soluble amine-aldehyde developed by reaction of an amine fraction with the cephalin.[12] There is a correlation between palatability and either ultraviolet absorption or fluorescence. On oxidative degradation of

[12] B. G. Edwards and H. J. Dutton, *Ind. Eng. Chem.* 37, 1121-2 (1945).

dried egg powder there is a correlation between palatability and lipid fluorescence.[13] The same sample serves for estimation of carotenoids.

Sample—*Dried eggs.* This method gives an extract suitable for estimation both of carotenoids and of lipid-amine aldehydes. Since the latter is fluorimetric, the reagents require precautions to avoid extraneous effects. All glassware must be cleaned with bichromate-sulfuric acid, thimbles ether-extracted for 2 hours, and ether refluxed and distilled from lead-sodium alloy. Extract 1 gram of dehydrated egg with anhydrous ether in a micro-Soxhlet for 4 hours. Dilute the solution to 25 ml. for reading carotenoids (Vol. III, page 37) and use for the lipid-amine fluorescence.

Procedure—*By fluorescence.* Dilute 1 ml. of the ether extract to 10 ml. with ether and read the fluorescence when excited by the 365 mμ line of mercury, using Coleman filters B_1 and PC_1 against a blank with 0.4 microgram of quinine sulfate per ml. in 1:360 sulfuric acid as fluorescence standard.

By ultraviolet absorption. Read at 270 mμ against a reagent blank.

Methylamine

The reaction of monomethylamine in alkaline solution to give a red color with lactose is appropriate for its estimation.[14] The color increases over a long period of time. Ammonia increases the sensitivity of the readings and should be standardized. Dimethylamine and trimethylamine in large concentrations inhibit color development. Urinary pigments do not interfere.

Sample—*Urine.* If the concentration of monomethylamine is of the order of 0.1 mg. per ml., use 0.1 ml. without distillation. At 0.01–0.1 mg. per ml. of methylamine nitrogen, steam-distil 10 ml. in a microdistillation apparatus in the presence of 5 ml. of 30 per cent sodium hydroxide. Add a few drops of mineral oil to reduce foaming. Receive in 10 ml. of 1:60 hydrochloric acid and, if the acid reaction to nitrazine paper tends to disappear, add a few drops of 1:5 hydrochloric acid. Steam-distil for 10 minutes, then for 1 minute with the acid below the

[13] H. J. Dutton and B. G. Edwards, *Ind. Eng. Chem.* 37, 1123-6 (1945); *Ind. Eng. Chem., Anal Ed.* 18, 38-41 (1946).

[14] Andrew A. Ormsby and Shirley Johnson, *J. Lab. Clin. Med.* 34, 562-5 (1949); *J. Biol. Chem.* 187, 711-17 (1950).

tip of the condenser, and finally for 1 minute without water in the condenser. Neutralize the distillate to pH 6-7, dilute to 50 ml., and use aliquots.

At less than 0.01 mg. of methylamine nitrogen per ml. acidify 50 ml. of urine with 1:1 hydrochloric acid, concentrate on a water bath to 10 ml., and treat as a sample containing 0.01–0.1 mg. per ml. but distil into 10 ml. of 1:5 hydrochloric acid.

Procedure—Dilute a sample containing 0.01–0.15 mg. of methylamine nitrogen to 6 ml. Add 0.5 ml. of 6.607 per cent ammonium sulfate solution neutralized to litmus and preserved with chloroform, less any known correction for ammonia in the sample solution. Add 0.5 ml. of 3 per cent lactose solution followed by 0.2 ml. of 20 per cent sodium hydroxide solution. Mix and incubate without evaporation at 56° for 30 minutes. Read at 540 mμ against a reagent blank.

1-Phenyl-2-Methylaminoprfpanol, Ephedrine

Ephedrine reacts with ninhydrin in alkaline solution to give a violet color.[15] The developed color is extractable with amyl alcohol, thus giving a good control on the concentration for reading. The color either disappears on dilution or does not pass readily into the amyl alcohol layer with emetine, apomorphine, and dilaudid. No color is given by alypine, aneurine, antipyrine, brucine, bulbocapnine, caffeine, metrazole, choline, cocaine, colchicine, codeine, cicutine, dicodid, dionine, eucodal, eupaverine, harmine, heroine, homatropine, hydrastinine, morphine, narceine, narcotine, orthoform, papaverine, pantocaine, pilocarpine, pyramidone, stovaine, theobromine, quinine, quinidine, scopalamine, scuroform, sparteine, strychnine, veratrine, and yohimbine.

Ephedrine oxidized in alkaline solution with hydrogen peroxide gives a violet color.[16] There is no interference by barbital, calcium chloride, codeine, magnesium chloride, potassium sulfoguaiacolate, terpin hydrate, or phosphates. Lactose, lanolin, and sugars interfere. This alkaloid is also determinable by its amine properties in reaction with picric acid. Precipitation as the reineckate is not complete, preventing the application of that method. Ephedrine is determined in benzene by its reaction with picryl chloride.[17] Determinations agreed with the

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[15] H. Wachsmuth, *J. pharm. Belg.* **4**, 186 (1949).

[16] Antonio Capone, *Boll. chim. farm.* **90**, 465-72 (1951).

[17] L. F. Chatten and L. I. Pugsley, *J. Am. Pharm. Assoc.* **41**, 108-10 (1952).

amounts in commercial products except ointments, as labeled by the manufacturer. Other compounds containing the same chemical groups may interfere.

Sample—*Aqueous isotonic sprays and water-soluble jellies.* Make a sample containing 50–100 mg. of ephedrine hydrochloride alkaline to litmus with 20 per cent sodium hydroxide solution, and add 0.5 ml. in excess. Extract with six 15-ml. portions of benzene. Combine the extracts and make up to 100 ml. with benzene. Remove an aliquot equivalent to 0.4 mg. of ephedrine and make up to 9 ml. with benzene. Develop with picryl chloride.

Syrups. Proceed as with isotonic sprays. If emulsions are formed during the benzene extraction, add 10 ml. of water to a sample containing 50–100 mg. of ephedrine hydrochloride. Make alkaline with 20 per cent sodium hydroxide solution and extract with six 15-ml. portions of ether or until extraction is complete. Combine the ether portions and extract with 5 ml. of 10 per cent sulfuric acid and then with four 5-ml. portions of water. Proceed as for isotonic sprays, from "Make . . . alkaline to litmus . . . ."

Capsules. Add 10 ml. of water to 20 capsules. Wash the empty capsules with another 10 ml. of water, filter, and add the filtrate to the first solution. Continue as for isotonic sprays, from "Make . . . alkaline to litmus . . . ."

Tablets. Powder 20 tablets and dissolve an amount containing 50–100 mg. of ephedrine hydrochloride in 20 ml. of water and filter. Wash the filter, and add to the filtrate about 500 mg. of anhydrous sodium carbonate and sufficient sodium chloride to make a saturated solution. Proceed as for isotonic sprays, from "Extract with six 15-ml. portions of benzene."

Oily nose drops. To an amount containing 50–100 mg. of ephedrine add 10 ml. of ether. Extract with 5 ml. of 10 per cent sulfuric acid and then with four 5-ml. portions of water. Combine the acid and aqueous extracts and make alkaline with 6–7 ml. of 20 per cent sodium hydroxide solution. Continue as for isotonic solutions, from "Extract with six 15-ml. portions of benzene."

Ointments. Dissolve an amount containing 10 mg. of ephedrine in ether, filter, wash the filter and continue as for oily nose drops, from "Extract with 5 ml. of 10 per cent sulfuric acid . . . ."

Procedure—*By ninhydrin.* Add 1 drop of 1 per cent ninhydrin solution and 1 drop of 1 per cent sodium carbonate solution to 2 ml. of the sample solution and warm. Extract the color completely with a volume of amyl alcohol selected according to the color intensity and read at 550 mμ.

By peroxide. To a sample containing 1–15 mg. of ephedrine, add 4 ml. of 16 per cent sodium chloride solution, 0.45 ml. of 0.4 per cent sodium hydroxide solution, and 7 drops of 30 per cent hydrogen peroxide. Dilute to 25 ml. and heat in boiling water for 5 minutes. Cool and read against a reagent blank.

By picryl chloride. To 9 ml. of sample solution in benzene add 1 ml. of 0.3 per cent picryl chloride in benzene. Stopper and place in a water bath at 75–77° for 20 minutes. Let stand at room temperature for 3 minutes, then read at 400 mμ against a reagent blank.

Dimethylamine

A complex version of the copper thiocarbamate reaction is applied to dimethylamine.[18] When shaken in aqueous alkaline solution with a 5 per cent solution of carbon bisulfide in benzene and a copper salt, the amber tint is measured. The solution of carbon bisulfide in benzene and aqueous solution of dimethylamine do not react unless ammonia or other material mutually soluble in both phases is present. Ammonium acetate serves to buffer ammonium ions. The stability of the color developed is increased by later complete removal of ammonia from the benzene layer.

Monomethylamine gives a similar color reaction which decreases in intensity as the alkalinity of the aqueous phase is increased. The method does not distinguish between dimethylamine and other secondary alkylamines. The intensity of the color obeys Beer's law, if the dimethylamine content is less than 0.05 mg. in the procedure given. No normal urinary constituents interfere in the determination.

This reaction is also applicable to determination of the possible content of up to 3 per cent of dimethylamine in commercial trimethylamine or monomethylamine.[19] Large amounts of monoethanolamine must be removed to avoid interference. Small amounts do not interfere

[18] Harry C. Dowden, *Biochem. J.* 32, 455-9 (1938).

[19] Edward L. Stanley, Harry Baum, and Jessie L. Gove, *Anal. Chem.* 23, 1779-2 (1951).

as the absorption of the thiocarbamate is at a wave length about 70 mμ lower. Methanol equal to the dimethylamine does not interfere. Recovery to ±1 per cent can be expected. The method for trimethylamine with picric acid (page 40) is also applicable to dimethylamine in the absence of interfering substances.

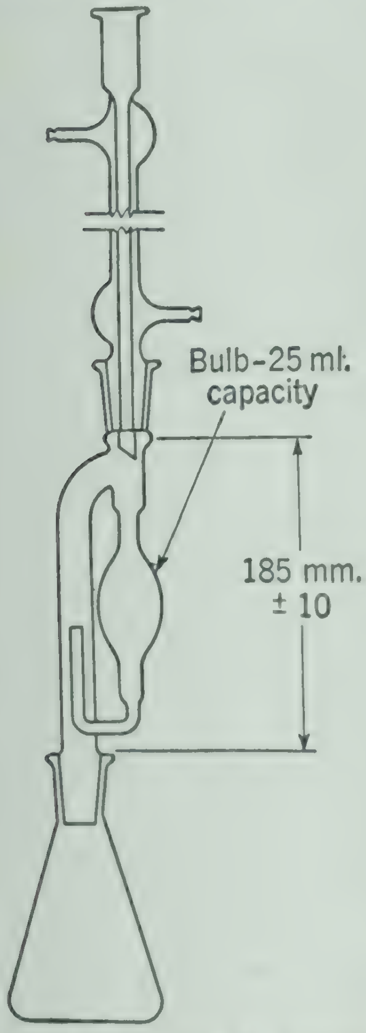

Fig. 3. Apparatus for separation of dimethylamine and monomethylamine

Sample—*Solutions.* Neutralize to phenolphthalein and adjust to 0.0006–0.015 mg. of dimethylamine per ml.

Urine. Distil a mixture of 5 ml. with 50 ml. of water and 10 ml. of 4 per cent sodium hydroxide solution. Collect 40 ml. of distillate, dilute to 50 ml., and use an aliquot as sample.

Trimethylamine. Weigh 25 ml. of sulfuric acid of known concentration, such as 1:17, and a drop of methyl red indicator. Add the sample of trimethylamine until a yellow endpoint is reached. Weigh to determine the sample used. Back titrate to pink with a drop or two of acid and dilute to a range of 0.001–0.005 mg. of dimethylamine per ml.

Monomethylamine. Weigh 50 ml. of 1:5 hydrochloric acid with a drop of methyl red indicator. Add monomethylamine until the solution turns yellow and weigh to get the sample weight. Back titrate to pink with a drop or two of 1:10 hydrochloric acid and dilute to 100 ml. Transfer 5 ml. to the flask of the apparatus shown in Figure 3 and add 50 ml. of chloroform. Place 10 ml. of chloroform in the trap, if not already charged, and reflux for one hour after a precipitate appears. This distils off the water as an azeotrope boiling at 56.1°, containing 2.5 per cent of water.[20] Thus precipitation of monomethylamine hydrochloride in the absence of water removes it from reaction. The dimethylamine

[20] L. H. Horsley, *Ind. Eng. Chem., Anal. Ed.* **19**, 508-600 (1947).

hydrochloride is in the chloroform. Every trace of water must be distilled. Remove the flask, stopper, and let cool to room temperature. Decant the chloroform solution through glass wool and wash the flask, precipitate, and funnel with chloroform. Dilute the chloroform solution to 100 ml. and add a 5-ml. aliquot to 10 ml. of water. Evaporate the chloroform without substantial distillation of water and use this aqueous solution as sample.

Procedure—As reagent dissolve 20 grams of ammonium acetate and 0.2 gram of copper sulfate pentahydrate in 30 ml. of water, add 10 grams of sodium hydroxide in 25 ml. of water and 20 ml. of concentrated ammonium hydroxide, and dilute to 100 ml. with water.

To 10 ml. of sample solution, add 1 ml. of reagent and 10 ml. of 5 per cent carbon bisulfide in benzene. Warm to 43–48°, stopper, and shake vigorously for 30 seconds. Add 1 ml. of 30 per cent acetic acid and shake again for 30 seconds. Cool and centrifuge to separate the solvent layer. Read at 434 mμ within an hour against a reagent blank.

dl-6-Dimethylamino-4,4-diphenyl-3-heptanone, Methadone

Methadone, ordinarily the hydrochloride, is estimated by its color with *m*-dinitrobenzene in ethanol.[21] It also reacts with most alkaloidal reagents.[22] Thus as a typical case it is precipitated with methyl orange at pH 6, the precipitate taken up in chloroform, and that solution extracted with 1:15 hydrochloric acid for reading at 490 mμ. Precipitation with Reinicke's salt from faintly acid solution, filtration, solution in acetone, and reading at 515 mμ is also applicable.

Procedure—Evaporate a sample containing about 5 mg. of methadone to dryness. Take up in about 1 ml. of ethanol and filter to remove sodium chloride. Wash the residue with not over 1 ml. of ethanol. To the filtrate add 4 ml. of 1 per cent *m*-dinitrobenzene solution in ethanol and 4 ml. of 4 per cent sodium hydroxide solution. Dilute to 10 ml. with ethanol. Incubate at about 45° for 10 minutes and let stand at room temperature. Read at 500 mμ against a reagent blank 1 hour after mixing.

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[21] J. S. Faber, *Pharm. Weekblad* **85**, 719-23 (1950).

[22] Bjarne Salvesen, *Medd. Norsk Farm. Selskap.* **14**, 1-11 (1952).

Trimethylamine

The yellow color characteristic of amine picrates in toluene solution is used to estimate trimethylamine in aqueous solution.[23] The method was developed for estimation of the degree of deterioration of fish. There is no interference by proteins, glycine, asparagine, cysteine, cystine, tryptophan, histidine, tyrosine, glutathione, creatine, creatinine, allantoin, urea, hydroxylamine, guanine, betaine, choline, and ammonia. Lecithin from fat interferes by giving a cloud in the developed solvent but is removed from the sample by extraction. Similar reactions are given by mono-, di-, trimethyl-, and ethyl-, and propylamine as well as indole and histamine. The first two are present to only small extents and the others not at all in fish before they are organoleptically spoiled. The general rule is that the picrate color is intensified by number and length of attached chains.

The reaction product of trimethylamine with *cis*-aconitic anhydride in toluene and acetic acid is violet and suitable for estimation.[24]

Sample—*Fish muscle.* Mince 5 grams and suspend in 15 ml. of water. At once add 20 ml. of 10 per cent trichloroacetic acid solution to precipitate the protein. Centrifuge when well coagulated and use an aliquot of the supernatant layer for development with picric acid.

Fatty fish. Mince 5 grams and suspend in 5 ml. of 1:100 sulfuric acid. Extract fat and lecithin by shaking with two successive 10 ml. portions of petroleum ether. Discard the extracts and neutralize the aqueous layer by careful addition of a little over 1 ml. of 10 per cent sodium hydroxide solution. Dilute with water to 15 ml. and proceed as for fish muscle starting at "At once add 20 ml. . . .."

Procedure—*By picric acid.* Dilute an aliquot of sample containing 0.002–0.02 mg. of nitrogen as trimethylamine to 4 ml. Shake commercial 37 per cent formaldehyde solution with magnesium carbonate, filter, and dilute with 3 volumes of water. Add 1 ml. of this, 10 ml. of toluene, and 3 ml. of 50 per cent potassium carbonate solution. Stopper with a cellophane-covered cork and shake 30–40 times to transfer the amines to the toluene layer. Shake a 5-ml. aliquot of the

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[23] Derek Richter, Margaret H. Lee, and Denis Hill, *Biochem. J.* **35**, 1225–30 (1941); W. J. Dyer, *J. Fisheries Research Board Can.* **6**, 351–8 (1945); W. J. Dyer and Yvonne A. Mounsey, *Ibid.* **6**, 359–67 (1945); W. J. Dyer, *Ibid.* **7**, 576–9 (1950).

[24] B. T. Chromwell, *Biochem. J.* **46**, 578–81 (1950).

solvent layer with 0.3–0.4 gram of anhydrous sodium sulfate and decant into 5 ml. of 0.02 per cent picric acid in dry toluene. Read the color at 410 mμ or in the ultraviolet at 345 mμ against a reagent blank. If free from ammonia the color is stable for a month.

By cis-aconitic anhydride. Shake a sample containing 0.01–0.1 mg. of trimethylamine and 3 ml. of saturated potassium carbonate solution with 10 ml. of toluene for 1 minute. Transfer the toluene solution and dry with about 0.5 gram of anhydrous sodium sulfate. Add 5 ml. of the dried solution to 0.1 ml. of 2.5 per cent *cis*-aconitic anhydride solution in glacial acetic acid and dilute to 10 ml. with toluene. Heat for exactly 10 minutes at 65° and cool. Read after 15 minutes at 420 mμ against a reagent blank.

β-Phenylethylamine, Tyramine

Tyramine may be visualized as decarboxylated tyrosine. As a phenol it gives many of the reactions of tyrosine. Thus it gives the characteristic color with *p*-phenyldiazonium sulfonate.[25] Practically any phenolic body interferes, necessitating separation of the tyramine. The yellow color with picric acid is also suitable for estimation in the absence of other amines giving the reaction.[26] This is described earlier as a general reaction (page 29).

A reaction with α-nitroso-β-naphthol to give a red color which fades after a few minutes is sensitive to 1 ppm. but only semiquantitative.[27]

Sample—*Protein.* Hydrolyze a 3-gram sample of vacuum-dried protein by refluxing for 24 hours with 40 ml. of water and 90 ml. of concentrated hydrochloric acid. All-glass apparatus is desirable.

Evaporate the residue to dryness *in vacuo* in 200 ml. of water. Boil on a steam bath with 100 ml. of saturated barium hydroxide solution for 1 hour. This drives off ammonia and precipitates humin. Filter on a folded filter or a Büchner funnel. Wash the residue with 10 ml. of saturated barium hydroxide solution. Add 1:1 sulfuric acid carefully until no further precipitation occurs. Digest, filter, and concentrate the filtrate to 30 ml. Add 50 ml. of 50 per cent sodium

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25 Milton T. Hanke and Karl K. Koessler, *J. Biol. Chem.* **50**, 235-69 (1922).

26 Derek Richter, *Biochem. J.* **32**, 1763-9 (1938); P. Dessi and C. Rizzoli, *Boll. soc. ital. biol. spec.* **24**, 1250-4 (1948).

27 P. Muller, *Compt. rend. soc. biol.* **123**, 128-30 (1936).

hydroxide solution and centrifuge. Decant the clear supernatant liquid and wash the residue with a mixture of 5 ml. of 50 per cent sodium hydroxide solution and 3 ml. of water. Discard the solid residue.

Combine the decantate and washings and extract with 25 ml., 10 ml., 10 ml., and 10 ml. of amyl alcohol. Discard the aqueous alkaline solution and combine the amyl alcohol extracts. Extract the amyl alcohol with two 50 ml. and two 25 ml. portions of 1:20 sulfuric acid. The histamine and tyramine and small amounts of amino acids are in the sulfuric acid solution. Add hot concentrated barium hydroxide solution until alkaline. Add 1:10 sulfuric acid drop by drop until a slight excess is present. Digest, filter, and wash the residue with 50 ml. of water. Concentrate the filtrate to 30 ml.

Add 50 ml. of 50 per cent sodium hydroxide solution and extract the histamine and tyramine with amyl alcohol as before. The amounts of amino acids extracted are negligible. Extract from the amyl alcohol with 1:20 sulfuric acid as before and precipitate sulfates with barium hydroxide. Concentrate to a semisolid condition and follow the technic described for tyrosine (page 124), starting at "Take up with 50 ml. of water and add 600 ml. of an 0.8 per cent solution of silver sulfate." Following through to the end, reserve the silver precipitate for separate determination of histamine. The final sample is purified tyramine for development by *p*-phenyldiazonium sulfonate.

Urine. To 10 ml. of urine add 10 per cent sodium hydroxide solution until distinctly alkaline and extract with 3 ml. of toluene. Remove the upper layer as completely as feasible and repeat the extraction. Mix the combined extracts, centrifuge to separate water, and use 3 ml. of the extract as sample for development with picric acid.

Procedure—*With p-phenyldiazonium sulfonate.* Follow the technic described for tyrosine (page 126) but use a curve developed from tyramine.

With picric acid. Use the general method (page 31).

4,4-Tetramethyldiaminodiphenylmethane, Tetra base

The yellow nitro derivatives formed by the reaction of tetramethyldiaminodiphenylmethane with nitrous acid in strongly acid solution is the basis of a method; most of the yellow color is attributed to *p*-nitrodimethylaniline.[28] Any compound that is extracted by hydro-

[28] S. Walter Denton, R. M. Oliver, and John T. Wiley, *Proc. Am. Petroleum Inst. III*, 29M, 68-71 (1949).

chloric acid and forms a color interferes. Thus dimethylaniline interferes, but aniline, quinoline, pyridine, and *p*-toluidine do not. Phenol interferes at concentrations above 1 per cent, but is removed by extraction with alkali. The method is consistently about 5 per cent low.

Sample—*Oil or grease.* Weigh an oil or grease sample containing 1–1.6 mg. of tetra base into 25 ml. of 200°-endpoint naphtha. If the sample contains more than 1 per cent of phenol, extract with 10 ml. of 0.4 per cent sodium hydroxide solution to remove phenol. Discard this extract. Extract the naptha solution with two 5-ml. portions of 1:120 hydrochloric acid, and one 5-ml. portion of water. Combine these extracts for use as sample.

Procedure—To the acid solution of sample add 2 ml. of 10 per cent sodium nitrite solution and 25 ml. of ethanol. Mix and dilute after 5 minutes to 100 ml. with water. Filter if turbid, discarding the first 5 ml. of filtrate. Read immediately at 420 mμ against a reagent blank.

β-Imidazolyl-4-ethylamine, Histamine

Structurally histamine is decarboxylated histidine. The reaction with *p*-phenyldiazonium sulfonate is used for its estimation after interfering substances have been removed.[29] The method is applicable to 0.01 mg. of the imidazole. By replacement of the sodium carbonate solution in the reaction by sodium hydroxide solution, the reaction is less subject to interference.[30] Accuracy is to ± 3 per cent down to 0.001–0.005 mg. Addition of ethanol after the diazo reagent stabilizes the color for about an hour.[31] An alternative is the use of *p*-nitrophenyldiazonium chloride made by diazotizing *p*-nitroaniline.[32] The resulting colored compound can be extracted from the mixture with methyl isobutyl ketone.[33] The sensitivity is approximately to 0.0005 mg.

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[29] Karl K. Koessler and Milton T. Hanke, *J. Biol. Chem.* **39**, 497-519 (1919). Milton T. Hanke, *Ibid.* **66**, 475-88 (1925); A. Maciag and R. Schoental, *Mikrochemie* **24**, 243-50 (1928); E. Havinga, L. Seckles and Th. Strengers, Jr., *Rec. trav. chim.* **66**, 605-10 (1947); G. Marac, *Bull. soc. chim. biol.* **32**, 287-9 (1950).

[30] Ryuhei Yokoyama, *Japan J. Med. Sci.* **VIII, 4**, No. 1; *Proc. Soc. Internal Med.* **31**, 208-10 (1936).

[31] A. Maciag and R. Schoental, *Mikrochemie* **24**, 243-50 (1928).

[32] Pietro Dessi and Tullio Franco, *Boll. soc. ital. biol. sper.* **25**, 1368-70 (1949).

[33] Sanford M. Rosenthaler and Herbert Tabor, *J. Pharmacol. Exptl. Therap.* **92**, 425-31 (1948).

Histamine reacts with 2,4-dinitrofluorobenzene to form a stable colored derivative, N-α-(2,4-dinitrophenyl)histamine.[34] Copper interferes, so that even water free from copper must be used. Phenol should be present only in very small amount and can be removed by extraction with benzene. The method is applicable to 0.001–0.008 mg. of histamine base per ml.

Histamine also gives a violet color in the presence of cobalt nitrate and sodium hydroxide.[35] The complex is sensitive to oxidizing and reducing agents. Dissolved air must therefore be removed for accurate results. Large amounts of ammonium salts interfere. Pilocarpine and anserine give no color. Imidazole dicarboxylic acid gives a faint rose precipitate. Imidazole gives a stable violet precipitate. No color is given by creatinine, tyramine, xanthine, hypoxanthine, and thymine. Guanine and adenine give a transient violet which soon disappears *in vacuo*.

On extraction of a trichloroacetic acid filtrate with ether, indole, purines, and pyrimidine are removed. Histamine is then extractable from the aqueous layer with amyl alcohol. Finally, the histamine is extractable from the amyl alcohol with 1:10 hydrochloric acid.[36] This is suitable for diazotizing and coupling.

The general color reaction of amino nitrogen compounds with 1,2-napthoquinone-4-sulfonate in alkaline solution to form highly-colored compounds[37] is applicable by the red color with histamine in the absence of other amines.[38] The method is rapid and accurate to ±3 per cent for samples up to 0.08 mg. Glycerin and phenol do not interfere, whereas ammonia and nitrogenous bases, such as the alkaloids and aminobenzenes, give positive reactions.

Sample—*Dry solids.* Mix 5 grams with about 50 ml. of 1:1 hydrochloric acid. Reflux on a sand bath for 30 hours. Evaporate the acid and water at 60° *in vacuo* and dry *in vacuo* at not over 80° for 1 hour. Dissolve the residue in 75 ml. of water and add 1 ml. of a thick lime slurry. Add 40 ml. of ethanol and evaporate to 50 ml. *in vacuo*

[34] Floyd C. McIntire, *J. Am. Pharm. Assoc., Sci. Ed.* **41**, 277 (1952).

[35] Walther Zimmermann, *Z. physiol. Chem.* **186**, 260-2 (1930).

[36] Jacques Baraud, Louis Genevois, Gabriel Mandillon and Guy Ringenbach, *Compt. rend.* 222, 760-1 (1946).

[37] Elizabeth G. Frame, Jane A. Russell, and Alfred E. Wilhelmi, *J. Biol. Chem.* **149**, 255-70 (1943).

[38] W. J. Mader, H. S. Sterne, Jr., J. Rosin and H. A. Frediani, *J. Am. Pharm. Assoc.* **39**, 175-6 (1950).

at 40°. This removes ammonia. To eliminate humins, filter on a Büchner funnel and wash the residue thoroughly with hot water.

Add hydrochloric acid to the filtrate until it is strongly acid to litmus. Evaporate to dryness on a water bath in glass. Dissolve the residue in about 100 ml. of water, containing 9 ml. of concentrated hydrochloric acid. Dissolve 20 grams of phosphotungstic acid in 250 ml. of water containing 9 ml. of concentrated hydrochloric acid per 100 ml. Heat the solution of hydrolyzed sample and phosphotungstic acid solution nearly to boiling and mix. Let cool slowly to room temperature. Then cool in a refrigerator for 24 hours and filter with suction. Wash the precipitate with an ice-cold solution containing 18 ml. of concentrated hydrochloric acid and 15 grams of phosphotungstic acid in 200 ml. The precipitate contains histamine, histidine, arginine, lysine, cystine, tyramine, and some other amines.

Suspend the precipitate in 500 ml. of hot water. Add an excess of a hot saturated solution of barium hydroxide. Heat for 1 hour on a water bath, cool, and filter on a Büchner funnel. Wash the precipitate with hot water. Heat the filtrate on a water bath and precipitate nearly all of the barium by careful addition of 1:35 sulfuric acid. Digest and filter on a folded filter. Evaporate the filtrate to dryness in glass. Dissolve the residue in the smallest possible volume of water and dilute to a known volume.

Mix 10 ml. of sample solution with 3 grams of solid sodium hydroxide and extract 6 times with 20-ml. portions of redistilled amyl alcohol. Combine the amyl alcohol extracts and extract 5 times with 10-ml. portions of 1:35 sulfuric acid.

Heat the acid extracts on a water bath and add saturated barium hydroxide solution until faintly alkaline. Filter while hot and evaporate the filtrate to dryness in glass. This residue contains all the histamine and small amounts of amino acids such as histidine. To eliminate such amino acids, dissolve the residue in 10 ml. of water and repeat the extraction exactly as for the original 10 ml. of sample solution. Finally dissolve the purified residue in water and dilute to 25 ml.

If desired, the histamine fraction may be carried through additional purification steps, which insure greater accuracy of the results. Dilute the solution of purified residue to 100 ml. Add 5 ml. of 20 per cent silver nitrate solution. Add 8 grams of barium hydroxide dissolved in 50 ml. of warm water. Filter on paper by suction. Wash the precipitate with 50 ml. of cold saturated barium hydroxide solution.

The filtrate must be clear. The histamine is all in the silver precipitate.

Suspend the silver precipitate in 50 ml. of water. Add 3 ml. of concentrated hydrochloric acid and sufficient sodium sulfate solution to precipitate the barium completely. Digest on a water bath for 1 hour and filter. Wash the precipitate with hot water. Neutralize the filtrate with 10 per cent sodium hydroxide solution and evaporate to 10 ml. The solution should be only faintly yellow. Dilute to a known volume, such as 25 ml.

A further purification in chloroform is optional. Evaporate the liquid to dryness *in vacuo*. Add 10 ml. of pure methanol and 0.5 gram of potassium hydroxide to the residue. Add 200 ml. of redistilled chloroform and place in a refrigerator for 15 hours. Filter through a small folded filter. Wash the precipitate with 200 ml. of hot chloroform. Add a few drops of concentrated hydrochloric acid to the chloroform extracts and evaporate to dryness *in vacuo*. Dissolve the residue in 10 ml. of water and redistil *in vacuo* to remove traces of methanol. Dissolve in water and dilute to 25 ml. for development with *p*-phenyldiazonium sulfate or cobalt nitrate.

Hydrated tissue. Take a sample to give about 5 grams of solids. Add ethanol to a concentration of about 75 per cent. Add a few drops of acetic acid and heat on a water bath 1-2 hours to extract free histamine and coagulate proteins. Cool, filter, and wash the residue with ethanol. Evaporate to dryness on a water bath. Continue with the residue from filtration and the dried extract, separately, as described for dry solids.

Blood. Mix 30 ml. with 50 ml. of 1:1 hydrochloric acid and proceed as for dry solids from "Reflux on a sand bath for 30 hours."

Blood serum. Let blood clot in a refrigerator. Centrifuge and separate 30 ml. of pale yellow serum as sample. Treat as the solution of dry solids from ". . . add 1 ml. of a thick lime slurry."

Injection solutions. Dilute so that the sample approximates 0.035–0.055 mg. of histamine or an equivalent amount of histamine phosphate in 5 ml. Develop with 1,2-naphthoquinone-4-sulfonate.

Pharmaceutical products. Prepare a carbonate buffer of about pH 10.2 by mixing equal volumes of 10.6 per cent sodium carbonate solution with 8.4 per cent sodium bicarbonate solution. Prepare a sample containing 0.02-0.1 mg. of histamine base per ml. in a 1:10 dilution of the above containing 0.07 per cent of sodium diethyldithiocarbamate. Determine by 2,4-dinitrofluorobenzene.

Procedure—*By p-phenyldiazonium sulfonate.* As reagent, mix 4.5 grams of sulfanilic acid with 45 ml. of concentrated hydrochloric acid and dilute to 500 ml. The solid dissolves slowly but completely. Dissolve 22.5 grams of sodium nitrite in water and dilute to 500 ml. Mix 1.5 ml. each of the sulfanilic acid and sodium nitrite solutions in an ice bath. After 5 minutes, add 6 ml. more of the sodium nitrite solution, mix, and let stand in the ice bath for 5 minutes. Dilute to 50 ml. and keep in the icebath. Do not use for at least 15 minutes after diluting. This reagent gives correct results after 24 hours. When the reagent is mixed with alkali a pale yellow color gradually develops.

Transfer 1-x ml. of water and 5 ml. of a 1.1 per cent solution of pure sodium carbonate to a comparison tube. Note the time to the second and add 2 ml. of reagent in 5 seconds. Mix by inclining the tube. Add x ml. of sample solution, containing 0.0002-0.005 mg. of histamine, exactly 1 minute after the reagent began to mix with the alkali. Mix as before. Read at 500 mμ against a reagent blank.[39]

By 2,4-dinitrofluorobenzene. To 1 ml. of sample solution add 1 ml. of reagent containing 0.15 ml. of 2,4-dinitrofluorobenzene in 25 ml. of alcohol. The reagent solution should be made fresh each week. Mix, and after 20 minutes, dilute the sample solution to 20 ml. with 1:160 hydrochloric acid. Extract the acidified solution with an equal volume of benzene. Read the aqueous phase at 358 mμ against a reagent blank.

By cobalt nitrate. To 1 ml. of sample containing about 2 mg. of histamine per ml. add 3 drops of 1 per cent cobalt nitrate solution. Add 10 drops of 8 per cent sodium hydroxide solution. Mix and read the color against a reagent blank. In the absence of histamine, only a faint blue due to basic cobalt compounds results.

By 1,2-naphthoquinone-4-sulfonate. To 5 ml. of sample add 1 ml. of 1 per cent sodium tetraborate solution. Mix and add 1 ml. of 0.5 per cent aqueous reagent, less than 8 hours old. Mix and place in boiling water for 10 minutes. Cool at 4-10° for 5 minutes. Add 1 ml. of a solution composed of 45 ml. of 1:10 hydrochloric acid and 10 ml. of 40 per cent formaldehyde all diluted to 80 ml. with water. Then add 1 ml. of 2.5 per cent sodium thiosulfate solution to decolorize excess reagent. Dilute to 10 ml., mix, and set aside for 30 minutes. Read at 460 mμ against a reagent blank. The factor for conversion of histamine to histamine acid phosphate is 2.76.

[39] Erik Jorpes, *Biochem. J.* 26, 1507-11 (1932).

3-Diethylamino-2,2-dimethyl Tropate

After separation, 3-diethamino-2,2-dimethyl tropate is nitrated and read.[40]

Procedure—Make 5 ml. of sample, expected to contain 2–3 mg. of test substance, alkaline by addition of 1:1 ammonium hydroxide. Extract successively with 10, 5, and 5 ml. of ether. Extract the combined ether extracts successively with 10, 10, and 10 ml. of 1:160 nitric acid. Dilute these acid extracts to 50 ml. Mix 2 ml. of the extract with 10 drops of fuming nitric acid and evaporate to dryness on a steam bath. Take up the residue in 2:98 absolute ethanol and acetone. Dilute to 10 ml. with the solvent and add 5 drops of 3 per cent potassium hydroxide in methanol. Read at 570 mμ within 5 minutes.

3,4,5-Trimethoxyphenylethylamine

The yellow color of picric acid with 3,4,5-trimethoxyphenylethylamine is suitable for reading.[41] Follow the general procedure (page 31).

Phosphatidylethanolamine, Cephalin

The complex phosphate cephalin is hydrolyzed to an ammonium salt and determined by treatment with sodium hypochlorite.[42]

Sample—*Nerve tissue.* Soak a finely ground weighed sample of nerve tissue for 24 hours with a known volume of ethanol. Centrifuge and dilute an aliquot to 0.4-1.5 mg. of test substance per ml.

Procedure—Heat a 1-ml. aliquot of prepared sample with 2 ml. of 1:5 hydrochloric acid in a hot water bath for 3 hours. Cool and dry *in vacuo* over sodium hydroxide. Extract the residue with 5 ml. of anhydrous ether to remove fatty acids. Centrifuge and retain the ether-free residue. This contains the ethanolamine as a salt. Add 6 ml. of concentrated hydrochloric acid and evaporate to dryness to convert to an ammonium salt. Take up in 0.5 ml. of water.

As a buffer dissolve 7.32 grams of boric acid and 2 grams of sodium hydroxide in water and dilute to 1 liter. Add 0.5 ml. of buffer to the

[40] Teodor Canbäck, *Farm. Rev.* **45**, 665-6 (1946).
[41] P. Dessi and C. Rizzoli, *Boll. soc. ital. biol. spec.* **24**, 1250-4 (1948).
[42] P. V. Edman and S. E. G. Aquist, *Acta Physiol. Scand.* **10**, 144-9 (1945).

neutral sample and then 0.5 ml. of 0.5 per cent chlorine water adjusted to pH 10 with 4 per cent sodium hydroxide. After this treatment with sodium hypochlorite, dilute to 5 ml. and heat in boiling water for 5 minutes. Read at 610 mμ against a reagent blank.

Indole ethylamine, 3-(2-aminoethyl)-indole, Tryptamine

Tryptamine after isolation gives a distinctive color with either dinitrobenzaldehyde or dioxane.[43] Alternatively, for determination by ferric chloride in the presence of trichloroacetic acid, follow the technics for β-indolylacetic acid (Vol. III, page 341).

Procedure—*Blood serum.* Treat the serum with a 1:1 mixture of ethanol and acetone containing 0.1 ml. of concentrated hydrochloric acid per 100 ml. Filter and evaporate the filtrate to dryness. Take up in ethanol and dry with excess of solid mercuric acetate. Take up the new residue in 1:1 ethanol and water, neutralize, and dry. Dissolve the residue in 10 ml. of 85 per cent phosphoric acid in the presence of 1 ml. of 1 per cent *m*-nitrobenzaldehyde or dioxane. Read against a reagent blank.

Piperidine-β-carboxylic Acid Diethyl Amide, Coramine

The familiar reaction with cyanogen bromide and benzidine is applied to coramine for estimation.[44]

Procedure—Dilute a sample solution containing 0.25–0.5 mg. of coramine to 1 ml. and add 0.5 ml. of a 4 per cent cyanogen bromide solution. Heat for 1 minute at 90° and add 1 ml. of 0.5 per cent benzidine in 10 per cent acetic acid. Shake for 5 minutes and dilute to 10 ml. with 70 per cent ethanol. Read within an hour against a reagent blank.

o-Dihydroxyphenylpropanolamine Hydrochloride, Corbasil

The method of oxidation of epinephrine with iodine is also applicable to corbasil.[45] Procaine does not interfere.

Procedure—Dilute an aqueous sample containing 0.1–1 mg. of corbasil to 25 ml. and add 5 ml. of 0.7 per cent solution of disodium

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[43] A. Lesure, *Union pharm.* **1939**, 122-3.
[44] Juan A Sánchez, *Semana méd.* (Buenos Aires) **1944, I,** 1269-73.
[45] Knud. A. Jackerott, *Dansk. Tids. Farm.* **15,** 217-35 (1941).

phosphate dodecahydrate and 1 ml. of 1:25 hydrochloric acid. Mix and add 1 ml. of 1.27 per cent iodine solution. Mix and at once add 2 ml. of 2.48 per cent sodium thiosulfate solution. Dilute to 50 ml. and read at 529 mμ against a blank from which the iodine was omitted.

β-Phenylisopropylamine, *dl*-α-Methylphenethylamine, Benzedrine, Sympamine, Amphetamine

Benzedrine is determined in physiological materials by extraction, distillation of the extract,[46] and development of a red dye by diazotization.[47] The method will determine as little as 0.03 mg. of β-phenylisopropylamine in 25 grams of sample with an average accuracy of about ± 5 per cent. Nicotine and pyridine do not interfere.

The general color of alphatic amines with picric acid is also applied.[48] The reaction is far from specific and, as originally applied to urine, gives a substantial reading due to other amines normally present.

Sample—To 25 grams of blood, urine, or finely minced tissue add 150 ml. of water, shake for 5 minutes, and let stand for 10 minutes longer. Add 10 ml. of 10 per cent sodium hydroxide solution and shake for 5 minutes. Add 30 ml. of 10 per cent sodium tungstate solution, shake well, then slowly add 30 ml. of 1:35 sulfuric acid with continuous shaking. Finally acidify with 1:1 sulfuric acid to precipitate proteins and add 5 ml. in excess. Let stand for 15 minutes and filter.

Neutralize the filtrate with 10 per cent sodium hydroxide and add 1 ml. in excess. Steam-distil, collecting 200 ml. of distillate in 10 ml. of 1:8 sulfuric acid. Neutralize the distillate with 10 per cent sodium hydroxide solution and add 1 ml. in excess. Extract by shaking for 1 minute successively with four 30-ml. portions of ether. Wash the combined ether extracts with 25 ml. of water and discard the washings. Extract the ether solution with three 10-ml. portions of 1:70 hydrochloric acid. Evaporate the combined acid extracts to dryness and take up the residue with 1 ml. of water for development by a diazo reagent.

For development with picric acid, measure out a sample of solution containing up to 0.1 mg. of benzedrine. Make distinctly alkaline by

[46] Wm. D. McNally, W. L. Bergman, and J. F. Polli, *J. Lab. Clin. Med.* 32, 913-17 (1947).

[47] Karl H. Beyer and J. T. Skinner, *J. Pharmacol, and Exptl. Therap.* 63, 419-32 (1940).

[48] Derek Richter, *Lancet* 1938, I, 1275; P. Dessi, *Farm. sci. e tec.* (Pavia) 5, 32-8 (1950).

addition of 4 ml. of 8 per cent sodium hydroxide solution. Agitate the solution with 6 ml. of petroleum ether to extract the benzedrine, and centrifuge. Use the clear solvent layer as sample.

Procedure—*By diazo reagent.* As a diazo reagent, chill 5 ml. of 0.05 per cent *p*-nitroaniline hydrochloride solution, mix with 1 ml. of concentrated hydrochloric acid, and chill in an ice-salt mixture for 10 minutes. Add 3 ml. of 0.7 per cent sodium nitrite solution and keep in the ice-salt mixture for 6 minutes. Dilute to 100 ml. with water at room-temperature, mix, and cool in the ice-salt mixture for 10 minutes. This reagent is stable at 0-4° for two weeks.

To 1 ml. of sample solution add 5 ml. of diazo reagent and then 5 ml. of 1.1 per cent sodium carbonate solution drop by drop with stirring. After 15 minutes, add 1 ml. of 10 per cent sodium hydroxide solution dropwise with stirring and let stand 10 minutes for color development. Dilute to 40 ml. and mix. Extract the colored compound with 10 ml. of *n*-butanol. If necessary, add a small quantity of sodium chloride to facilitate separation of the two layers. Read at 530 mμ against a reagent blank.

By picric acid. To 3 ml. of the benzedrine solution in petroleum ether, containing not over 0.05 mg. of benzedrine, add 3 ml. of chloroform and 0.6 ml. of a 1 per cent solution of picric acid in toluene. Let stand for 12 hours for foreign picrates to precipitate and read at 445 mμ against a reagent blank.

N-Pantoyl-3-propanolamine, Panthenol

Panthenol is α,γ-dihydroxy-β,β-dimethylbutyramide, one of the vitamin B complex. By cleavage to β-alanine the latter is estimated by reaction to give an orange-red color with 1,2-naphthoquinone-4-sodium sulfonate [49] or 2,4-dinitrophenylhydrazine.[50] Cleavage of panthenol and pantothenates in acid media yields pantoyl lactone and β-alanol or β-alanine respectively. The pantoyl lactone forms its hydroxamic acid with hydroxylamine in alkaline solution. This gives a purple complex with ferric chloride in acid solution.[51] The color starts to fade after a

[49] R. Crokaert, *Arch. intern. physiol.* **56**, No. 2, 189-91 (1948).

[50] C. R. Szalkowski, W. J. Mader, and H. A. Frediani, *Cereal Chem.* **28**, 218-25 (1951).

[51] Fritz Feigl, V. Anger, and O. Frehden, *Mikrochemie* **15**, 9-22 (1934); S. Hestrin, *J. Biol. Chem.* **180**, 249-61 (1949); Ernest G. Woolish and Morton Schmall, *Anal. Chem.* **22**, 1033-7 (1950).

minute. The average deviation is ±3 per cent. There is no interference from thiamine hydrochloride, riboflavin, niacinamide, biotin, or folic acid before or after hydrolysis. Pyridoxine hydrochloride gives a slight brownish color before or after hydrolysis, corrected by the blank. Ascorbic acid interferes. By sorption on resin, panthenol is separated from panthenates. Also consult the next topic, pantothenic acid, for further details.

Sample—*Water-soluble multivitamin preparations.* Place plugs of fiber glass over the stopcocks of three 14 × 200 mm. columns and fill to 40 mm. with Amberlite IRA-400-OH (Rohm and Haas). Wash each column with about 50 ml. of 1:4 hydrochloric acid and then with about 25 ml. of water. Add 50 ml. of 10 per cent sodium hydroxide solution, withdraw it, and wash thoroughly with about 50 ml. of water. Add water so that about 10 ml. is above the resin and stir to remove bubbles. Put a plug of fiber glass on top of each column and drain the water to about 5 mm. above this plug. The columns may be reused by similar treatment.

Add an aliquot of solution, not over 3 ml., equivalent to 2-3 mg. of panthenol to each of 3 columns. To the second column add 1 ml. of standard containing 1 mg. of panthenol and, to the third, 2 ml. of the same standard. Allow to flow through the columns at 0.5 ml. per minute until no liquid remains on top. Then elute with 80 ml. of water at 3 ml. per minute. Evaporate the eluates to 10 ml. on steam baths with an air stream and develop. Any pantoyl lactone or pantothenic acid is retained by the column.

Oil-base multivitamin capsules. Combine capsules containing about 40 mg. of panthenol by cutting with a razor blade. Add 50 ml. of petroleum ether and exactly 25 ml. of water. Shake mechanically for 1 hour. Separate the aqueous layer and centrifuge for 5 minutes. Filter 10-15 ml. of the aqueous layer and use 2-ml. aliquots by the method for water-soluble preparations.

Procedure—Dilute a sample containing about 3 mg. of panthenol or pantothenate to 5 ml. with water, or use the prepared sample which may total 10 ml. Add 3 ml. of 1:10 hydrochloric acid, stopper loosely, and heat at 80° for 3 hours. Cool and add 2 ml. of a fresh 7.5 per cent solution of hydroxylamine in 4 per cent sodium hydroxide solution. After 5 minutes add 3 drops of a 0.1 per cent solution of 2,4-dinitrophenol in ethanol as indicator. Titrate to colorless with 1:10 hydro-

chloric acid and dilute to 50 ml. Mix 5 ml. with 1 ml. of 2 per cent ferric chloride solution and read 45-60 seconds later at 500 mμ against water. Subtract a blank prepared without hydrolysis.

For the standard described under sample, calculate by $(A \times D)/[(B + C) - 2A \times E]$ = mg. of panthenol per ml. of sample solution in which

A = reading of sample solution
B = reading of sample plus 1 ml. of panthenol standard
C = reading of sample plus 2 ml. of panthenol standard
D = mg. of panthenol added to B and C
E = ml. aliquot of sample solution used.

Pantothenic Acid

Pantothenic acid is discussed in part under the preceding topic, panthenol. As a treatment after cleavage,[52] oxidize the β-alanine with potassium permanganate in the presence of potassium bromide to give an insoluble hydrazone with 2,4-dinitrophenylhydrazine. To estimate the latter dissolve in pyridine and dilute with aqueous sodium hydroxide. There is no interference by acetic, lactic, tartaric, glycolic, and succinic acids, α-alanine, ethanol, or riboflavin. Compounds which do interfere are carbohydrates, ascorbic acid, thiamine hydrochloride, pyridoxine hydrochloride, niacin, niacinamide, and soya flour. All of the interferences except ascorbic acid are eliminated by chromatographing.

Sample—*Feed enrichment mixtures.* Shake a weighed amount of sample containing about 200 mg. of calcium pantothenate with 200 ml. of water for 10 minutes. Dilute to 250 ml., mix thoroughly, and centrifuge. Prepare a 12 × 500 mm. chromatographic column with 8 grams of aluminum oxide and activate just before use by washing with 50 ml. of 1:1 hydrochloric acid followed by 100 ml. of water.

Pass an aliquot containing about 25 mg. of calcium pantothenate through the column and wash with successive portions of water, totaling 100 ml. Elute the column with 50 ml. of 1:1 sulfuric acid and then 10 ml. of water. Reflux the combined eluate and washings for 1 hour, cool to room temperature, and dilute to 250 ml. with water for development with pyridine.

[52] C. R. Szalkowski, W. J. Mader and H. A. Frediani, *Cereal Chem.* **28**, 218-25 (1951).

Procedure—To a 25-ml. aliquot of acid hydrolyzate in about 40 per cent sulfuric acid, add 5 ml. of 1:1 sulfuric acid and adjust the temperature to 22-25°. Add 5 ml. of 12 per cent potassium bromide solution and 10 ml. of 5 per cent potassium permanganate solution. Leave at 22-25° for 10 minutes, then cool in an ice bath for 5 minutes. Add 20 per cent sodium sulfite solution dropwise to decolorize the potassium permanganate. Add 10 ml. of 0.5 per cent 2,4-dinitrophenylhydrazine in 1:4 hydrochloric acid to the clear, colorless solution. Mix the precipitate and solution thoroughly, heat on a steam bath for at least 15 minutes, and cool to room temperature. Transfer the yellow precipitate to a sintered glass filter and wash with five 5-ml. portions of water. Dry at 100° for 30 minutes. Meanwhile, drain the precipitation flask and take up the traces of precipitate in two 3-ml. portions of hot pyridine. Transfer the pyridine washings to a 25-ml. flask. Fit the sintered glass filter to a filtration flask containing the 25-ml. flask. Add small portions of boiling pyridine to the filter and triturate the contents with a glass rod. Use suction to draw the resultant pyridine solution through into the flask. Dilute to 25 ml.

To a 5-ml. aliquot add 50 ml. of water and 5 ml. of 20 per cent sodium hydroxide solution. Dilute to 100 ml. and read at 570 mμ against a reagent blank. The color is stable for an hour.

Hydrazine

The orange of the azine formed by hydrazine with *p*-dimethylaminobenzaldehyde is a stable reaction product [53] which is soluble in mineral acids and develops the maximum color within 15 minutes. Many primary aromatic amines interfere. Urea, semicarbazide, and adrenaline give a slight reaction. Ammonium ion does not. The optimum range is at 0.006-0.047 mg. per 100 ml. of sample. Error is under ±1 per cent. The color develops at once, is stable after 10 minutes, and does not change for 12 hours. Hydrochloric acid over 1:15 increases the intensity of absorption.

Procedure—To 5 ml. of sample containing 0.0001-0.001 mg. of hydrazine per ml., add 1 ml. of reagent containing 0.4 gram of dimethylaminobenzaldehyde, 20 ml. of absolute ethanol, and 2 ml. of concentrated hydrochloric acid. Read after 15 minutes at 458 mμ against a reagent blank.

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[53] M. Pesez and A. Petit, *Bull. soc. chim. France* 1947, 122-3; George W. Watt and Joseph D. Crisp, *Anal. Chem.* 24, 2006-8 (1952).

Hydroxylamine

Hydroxylamine reacts with benzoyl chloride to give benzylhydroxamic acid. This forms a reddish-violet ferric salt in neutal or slightly acid solution.[54] Substances which react with hydroxylamine in the cold or which react with ferric ion must be absent. Glucose does not interefere if its concentration is not more than twenty times that of the hydroxylamine. With a sample concentration of 0.2-1 mg. the error is ± 2 per cent. The blue with chlorinated urea is extracted for estimation.[55]

Sample—*Solutions.* Add 1:1 hydrochloric acid or 10 per cent sodium hydroxide to a 10-ml. sample with cooling until the color of phenolphthalein is just discharged. Dilute to a known volume at which it will contain 0.2-1 mg. of hydroxylamine per ml., expressed as the hydrochloride.

Procedure—*By benzoyl chloride and ferric chloride.* To 4 ml. of sample solution add 2 drops of colorless benzoyl chloride, followed by 4 ml. of ethanol and 2 ml. of a 2 per cent solution of sodium acetate trihydrate. Add 2 ml. of a 0.5 per cent solution of ferric chloride hexahydrate in 1:50 hydrochloric acid and dilute to a known volume. Allow to stand for several minutes so that undissolved benzoyl chloride will settle. Read against a reagent blank.

By chlorinated urea. Prepare the reagent by saturating 60 grams of urea in 15 ml. of water with 30-32 grams of chlorine. The mixture of syrup and crystals decomposes slowly at 20° but can be kept for several weeks. To a 10-ml. sample add 2 ml. of 10 per cent sodium acetate trihydrate solution and two drops of cyclopentanone. Mix well and add 1 ml. of a fresh 10 per cent solution of the reagent. Shake and add 5 ml. of xylene. When the blue color is extracted, read the upper layer against a reagent blank.

N-(α-Pyridyl)-N-(p-methoxybenzyl)-N′,N′-dimethylethylenediamine, Pyranisamine Maleate, Neoantergan Maleate

Pyranisamine maleate is a nonproprietary antihistamine most satisfactorily read directly at 243.5 mμ.[56] A concentration of 2 mg. per

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54 Eug. Bamberger, *Ber.* **32**, 1805-6 (1899); George W. Pucher and Harold A. Day, *J. Amer. Chem Soc.* **48**, 672-6 (1926).

55 O. Wichterle and M. Hudlicky, *Collection Czechoslav. Chem. Communs.* **12**, 661-71 (1947).

56 Louise T. Anderson, W. C. Gakenheimer, Charles Rosenblum and E. H. Smith, *J. Am. Pharm. Assoc.* **38**, 373-7 (1949).

100 ml. is appropriate.

Procedure—*Tablets.* Allow 0.5 gram of tablets to disintegrate in 100 ml. of water and dilute to 500 ml. Mix thoroughly, dilute a 10-ml. aliquot to 500 ml. with water, and read at 243.5 mμ interpreting from a standard in water.

Elixirs. Dilute with 20 per cent ethanol or water so that the content is 2 mg. of maleate per 100 ml. of solution. If there is interference with direct reading, pretreat another sample as follows. Dilute 10 ml. of elixir to 100 ml. and extract three times with 10-ml. portions of ethyl ether. Dilute the aqueous phase to a concentration of 2 mg. of the antihistamine per 100 ml. and read at 243.5 mμ.

Anhydrous petrolatum ointments. Dissolve 5 grams of ointment in 100 ml. of petroleum ether and extract five times with 25-ml. portions of water. Combine the extracts, dilute to 500 ml. with water, and mix thoroughly. Dilute a 10-ml. aliquot to 250 ml. with water and read at 243.5 mμ.

Anhydrous water-soluble ointments. Dissolve a 5-gram sample in water to make 500 ml. of solution. Dilute a 10-ml. aliquot to 250 ml. to approximate 2 mg. of antihistamine per 100 ml. Read at 243.5 mμ against the blank containing the ointment base.

Aqueous emulsion-type ointments. Dissolve and dilute a 5-gram sample as described for water-soluble ointment but use equal volumes of absolute ethanol and petroleum ether as solvent. Read against a blank at 243.5 mμ.

Parenteral solutions. Dilute to approximate 2 mg. per 100 ml. and read at 243.5 mμ.

Hexamethylenetetramine, Urotropine, Methenamine

Urotroprine can be converted back to formaldehyde and so determined. Retention at a proper pH will give the result, but distillation is preferable. While any formaldehyde method would appear suitable, those with phloroglucinol [57] and chromotropic acid [58] have been used

[57] R. J. Collins and P. J. Hanzlik, *J. Biol. Chem.* 25, 231-7 (1916); Alfred T. Shohl and Clyde L. Deming, *J. Urology* 4, 419-37 (1920).

[58] Fernando Montequi Diaz de Plaze, *Anales real soc. españ. fís. y quím.* 47B 135-42 (1951).

Procedure—*Urine.* Distil a measured volume of urine until half the original volume is present as distillate. This contains both the formaldehyde originally present as such and the hexamethylenetetramine converted to formaldehyde.

Determine the formaldehyde in the original sample by phloroglucinol (Vol. III, page 258). Also use the distillate as a sample for estimation of formaldehyde. Subtract the free formaldehyde from the total formaldehyde given by this method and multiply by 0.781 to give the value of the balance in terms of hexamethylenetetramine.

Medicinals. Determine by chromotropic acid (Vol. III, page 259).

Glucosamine

Glucosamine acetate gives a red color with *p*-dimethylaminobenzaldehyde.[59] Mucin, after a short period of heating with dilute alkali, on acetylation shows the reaction due to the acetylated glucosamine present. The color which develops fades rapidly. Interference by urea, phosphate, and other urinary ingredients is negligible. Some sugars interfere, but the optimum for reaction of glucosamine is pH 9.5, while that for the sugars is 10.8-11.2.[60] Also glucosamine after heating in sodium carbonate solution no longer gives the red color with the reagent. The reaction will detect 0.05 mg. of glucosamine and is accurate to ±5 per cent on a sample of 0.5-5 mg.

Another method of application of the same reaction is by forming a pyrrol derivative.[61] Around pH 9.8, small changes have little effect on the stability of the color formed.[62] This is equally applicable to solutions of chondrosamine which give the same intensity of the reddish color as glucosamine. Glucose, galactose, fructose, arabinose, glycine, alanine, urea, glutamic acid, proline, arginine hydrochloride, lysine, serine, phenylalanine, leucine, tyrosine, hydroxyproline, valine, norleucine, and histidine do not interfere. Some pyrrol or indole deriva-

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[59] Fritz Zuckerkandl and Luise Messiner-Klebermass, *Biochem. Z.* **236**, 19-28 (1931); Torazo Miyazaki, *J. Biochem. (Japan)* **20**, 211-22 (1934); Walter T. J. Morgan and Leslie A. Elson, *Biochem. J.* **28**, 988-95 (1934).

[60] J. Immers and E. Vasseur, *Nature* **165**, 898-9 (1950).

[61] Leslie A. Elson and Walter T. J. Morgan, *Biochem. J.* **27**, 1824-8 (1933); Ivar Nilsson, *Biochem. Z.* **285**, 386-9 (1936); John W. Palmer, Elizabeth M. Smyth, and Karl Meyer, *J. Biol. Chem.* **119**, 491-500 (1937); Hajime Masamune and Yoshio Nagazumi, *J. Biochem.* (Japan) **26**, 223-32 (1937); Gunnar Blix, *Acta Chem. Scand.* **2**, 467-73 (1948).

[62] Benjamin Schloss, *Anal. Chem.* **23**, 1321-5 (1951).

tives interfere, but these give the color with the reagent in acid solution without prior treatment with acetylacetone. The color with tryptophan does not develop in the concentration of hydrochloric acid used, only in stronger acid. Many amino acids and carbohydrates also react at higher acidity. *n*-Acetylglucosamine or *n*-acetylchondrosamine react with the reagent to give about one-fifth the color intensity with a maximum at 530 mμ. The color with pyrrol has its maximum at 550 mμ. Variations in pH are critical. Substantially the same color is given by l-amino-glucose, but this is not likely to be present in acid hydrolysis products of glucoproteins or nitrogenous polysaccharides. Results are accurate to $\pm$0.5 per cent if the sample contains 0.012-0.83 mg. of glucosamine per ml. Prior treatment with acetylacetone can be replaced by treatment with ethyl acetoacetate, but the former causes a deeper color.

Hexosamines, deaminated by nitrous acid, yield 2,5-anhydrohexoses which, when reacted with indole in dilute hydrochloric acid, give characteristic colors.[63] A correction must be applied for the color developed with the sample before deamination. If the reaction mixture is shaken with chloroform, a pink color is taken up, and a brown color remains in the water phase. However, there is little difference in their absorption curves, and so separation is of little value. Nitrogen-free carbohydrates react with indole and hydrochloric acid and give a maximum absorption at 492 mμ, but this is much lower than that of deaminated hexosamines. Serum albumin at 0.2 per cent and ascorbic acid at 0.01 per cent, when treated with nitrous acid, show an absorption at 492 mμ in the indole reaction. Hence to avoid interference from these substances, readings are made at 520 and 492 mμ. The increase of this difference after deamination is a measure of the amount of hexosamine, as serum albumin and ascorbic acid do not show such an increase. Glucose and glucuronic acid do not interfere.

Hexosamines occur in polysaccharides in the form of their acetyl derivatives and determination of these hexosamines must be preceded by deacetylation by heating for 1 hour with 1:10 hydrochloric acid.

At 100°, buffered at pH 5.9, glucosamine reduces mercuric chloride rapidly and the acetate hardly reacts. Buffered at 7.4 glucosamine acetate reduces the reagent readily.[64] For quantitative application the

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[63] Zacharias Dische and Ellen Borenfreund, *J. Biol. Chem.* **184**, 517-22 (1950).

[64] L. Hahn, *Arkiv. Kemi. Mineral Geol.* **A22**, No. 12, 10 pp. (1946).

mercuric chloride unreduced is determined with diphenylcarbizone (Vol. II, page 73).

An alternative is to add saturated aqueous trisodium phosphate solution, saturated thereafter with borax.[65] This hydrolyzes the amine to ammonia for distillation into dilute boric acid solution and subsequent estimation by Nessler's reagent.

Samples—*Casein.* Hydrolyze 1 gram of casein by heating with an excess of 1:3.6 hydrochloric acid for 8 hours and evaporate to dryness *in vacuo.* Dissolve the residue in 50 ml. of water and add 1 gram of activated carbon. Heat to boiling and filter. Wash the residue thoroughly with hot water. Cool the filtrate and dilute to 100 ml. for the development of aliquots as the pyrrol derivative.

Proteins. Reflux 1 gram with 10 ml. of 1:1 hydrochloric acid for 5 hours. Neutralize with sodium carbonate and add 20 per cent of the volume of 20 per cent lead acetate solution. Dilute to 50 ml. Filter and precipitate lead from the filtrate with hydrogen sulfide. Extract with ether to remove acetic acid and develop an aliquot as the pyrrol derivative.

Ovomucoid. Heat 1 gram of mucin for 3 hours in 1:4 hydrochloric acid on a water bath and complete as for casein, starting at ". . . evaporate to dryness *in vacuo.*"

Alternatively, let the sample stand for 2 days at room temperature in concentrated hydrochloric acid and dilute with 3 volumes of water. Complete as for casein, starting at " . . . evaporate to dryness *in vacuo,*" but omit the carbon treatment.

Blood. Lake 2 ml. of oxalated blood with 2 ml. of water. Shake with 16 ml. of 4 per cent trichloroacetic acid solution to deproteinize. Let stand for 10 minutes and filter. Extract the filtrate with several successive 5-ml. portions of ether, discarding the extracts. Remove ether from the remaining aqueous solution by bubbling air through it and use as sample for development as the acetate.

Tissue. Grind 0.2-0.6 gram of tissue in a mortar. Transfer with 4-5 ml. of water, add 10 ml. of 7 per cent trichloroacetic acid solution, and dilute to 20 ml. Filter after 1 hour. Continue as for deproteinized blood, starting at "Extract the filtrate . . ." In the procedure evaporate to 0.5 ml. by heat and then continue to dryness *in vacuo* over phosphorus pentoxide.

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[65] M. V. Tracy, *Biochem. J.* **52**, 265-7 (1952).

Urine. To prepare urease, digest 1 part of soybean meal with 5 parts of water at room temperature for 1 hour, stirring occasionally. Filter or centrifuge and evaporate the extract to dryness at less than 1-mm. pressure. Powder the residue. Alternatively pour the extract into sufficient acetone so that it is dehydrated practically instantaneously, filter, and dry.

If alkaline, acidify with a few drops of 15 per cent trichloroacetic acid solution. Dilute 1 ml. to 7 ml. Add 0.3 gram of urease and 3 ml. of 4 per cent acid potassium phthalate solution. Heat at 40° for 1 hour to decompose urea. Add 1 ml. of 1:10 hydrochloric acid and 9 ml. of 10 per cent lead acetate solution. Mix and filter. Saturate the filtrate with hydrogen sulfide and filter off the lead sulfide on a Büchner funnel. Aerate to expel hydrogen sulfide and extract with several successive portions of ether to remove phthalic acid. Aerate to remove ether from the aqueous layer and use it as sample for development as the acetate. In the procedure evaporate the volume used to 0.5 ml. and dry *in vacuo* over phosphorus pentoxide.

Alternatively use a sample from which arginine was removed with Amberlite (page 157).

Procedure—*As the acetate with p-dimethylaminobenzaldehyde.* The acetate must first be formed. Evaporate a volume of sample to contain 1-5 mg. of glucosamine to dryness in a porcelain evaporating dish. Add 1 ml. of 5 per cent sodium methylate, not more than 2 days old. Rub the residue thoroughly with a glass rod and cool the dish. After 5 minutes, add 0.3 ml. of acetic anhydride dropwise. After 5 minutes add 1 ml. of water. Transfer to a filter and wash the residue first with 1 ml. and then with 0.5 ml. of water. To the filtrate add 1 ml. of ethanol and 0.5 ml. of 30 per cent sodium hydroxide solution. Let stand at 20° for 10 minutes. Heat at 100° for exactly 90 seconds, avoiding loss of water by evaporation. Cool at 20° for 10 minutes. Add 3 ml. of the pale-yellow, stable reagent which contains 0.8 gram of *p*-dimethylaminobenzaldehyde in 30 ml. of ethanol and 30 ml. of concentrated hydrochloric acid. Read at 535 mμ after 5 minutes correcting for both reagent and sample blanks.

As a pyrrol derivative with p-dimethylaminobenzaldehyde. Treat 2 ml. of sample containing 0.04-0.004 mg. of glucosamine per ml. with 5.5 ml. of a colorless reagent containing 0.9 per cent of acetylacetone and 4.8 per cent of sodium carbonate, with 1:1 hydrochloric acid added to pH 9.8. Heat for 20 minutes in boiling water which comes to above

the level in the container. Minimize evaporation. Cool and dilute to nearly 25 ml. with absolute ethanol. Add 2.5 ml. of a reagent containing 0.8 gram of *p*-dimethylaminobenzaldehyde in 30 ml. of absolute ethanol and 30 ml. of concentrated hydrochloric acid. Dilute to 25 ml. with absolute ethanol and incubate at 30° for 24 hours. Read at 512 mμ or 553 mμ against a reagent blank.

As anhydrohexoses. To 0.5 ml. of unknown, add 0.5 ml. of 5 per cent sodium nitrite solution, and 0.5 ml. of 1:2 acetic acid. Shake and after 10 minutes, which completes the amination, remove excess nitrous acid by adding 0.5 ml. of 12.5 per cent ammonium sulfamate solution. Shake repeatedly for 30 minutes. At the same time treat another 0.5 ml. of sample in the same way but do not let it stand, so that it will not be deaminated.

To 2 ml. of the solution which contains 0.005-0.1 mg. per ml. of deaminated hexosamine, and to 2 ml. of the one not deaminated, add 2 ml. of 1:20 hydrochloric acid and 0.2 ml. of 1 per cent solution of indole in ethanol. Immerse in a boiling water bath for 5 minutes. An intensive orange color and slight turbidity will appear. To remove the turbidity, add 2 ml. of ethanol and shake. Make readings at 520 and 492 mμ respectively. Subtract the value for $D_{492} - D_{520}$ of the nondeaminated sample from the other and read against a calibration curve.

Chondrosamine

The same pyrrol derivative and anhydrohexose are formed from chondrosamine as from glucosamine and estimated in the way just described.

n-Acetylglucosamine

In the enolic form, glucosamine acetate is 2-methyl-4-$\alpha,\beta,\gamma,\delta$-tetrahydroxy-$n$-butyl oxazole which reacts with *p*-dimethylaminobenzaldehyde.[66] n-Acetylchondrosamine gives an identical reaction. Accuracy to ± 2 per cent is obtained when sample and standard do not differ by more than 20 per cent. The color is sensitive to excess hydrochloric acid.

Procedure—As reagent dissolve 2 grams of *p*-dimethylaminobenzaldehyde in 95 ml. of glacial acetic acid and 5 ml. of concentrated hydrochloric acid. The final solution should be only pale yellow and addition of 1 ml. of water to 9 ml. of the reagent must not intensify this.

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[66] Walter T. J. Morgan and Leslie A Elson, *Biochem J.* **28**, 988-95 (1934).

Transfer a 1-ml. sample containing 0.1-1 mg. and add 0.1 ml. of 5 per cent sodium carbonate solution. Heat in boiling water for 5 minutes and cool. Add glacial acetic acid to about 8 ml. Add 1 ml. of reagent and dilute to 10 ml. with glacial acetic acid. Mix and let stand for 45 minutes. The color is then at a maximum and will not fade for an hour. Read against a reagent blank.

n-Acetylchondrosamine

This gives the identical reaction just cited for *n*-acetylglucosamine.

Ethyl Carbamate, Urethane

A tungstic acid blood filtrate, free from ethanol, can have its urethane content hydrolyzed with sodium hydroxide to liberate ethanol.[67] On distillation of the ethanol content it is oxidized by acid potassium dichromate (Vol. III, pages 47, 49).

Isopropyl-N-phenylcarbamate, IPC

The familiar oxidation of amines with hypochlorite in the presence of phenol is applicable to determination of isopropyl-N-phenylcarbamate.[68]

Sample—*Lettuce.* Disintegrate 300 grams in a blender for 2 minutes. Add 200 ml. of methylene dichloride and blend for 10 minutes, adding chopped ice to keep the temperature below 35°. Centrifuge and separate the solvent layer. Use 100 ml. more of solvent to rinse the sample and equipment. Filter and evaporate the solvent in a stream of air. Transfer to a flask with ether and evaporate the ether. Add 10 ml. of a 91:9 mixture of 85 per cent phosphoric acid and concentrated hydrochloric acid, and 5 ml. of glacial acetic acid. Reflux for one hour to hydrolyze. Cool and dilute to 50 ml. with water. Add 60 ml. of 25 per cent sodium hydroxide solution. Steam-distil through a still head, collecting 25-35 ml., and dilute to 50 ml. with water.

Procedure—Mix 10 ml. of sample solution with 2 drops of fresh 5 per cent calcium hypochlorite solution and 2 ml. of 12:78 hydrochloric

[67] Norwood K. Schaffer, Francis N. LeBaron, and B. S. Walker, *Proc. Soc. Exptl. Biol. Med.* **70**, 420-2 (1949).

[68] William E. Bissinger and Robert H. Fredenburg, *J. Assoc. Official Agr. Chem.* **34**, 812-16 (1951).

acid. After 5 minutes heat to boiling and add 5 ml. of fresh 5 per cent phenol solution in 1:19 ammonium hydroxide. Dilute with water to 20 ml. and after 15 minutes read against a reagent blank. Aniline can be used to prepare standards and results in terms of aniline multiplied by 1.93.

Quaternary Ammonium Compounds

The cations of quaternary ammonium compounds form colored salts in sodium carbonate solution with such indicators as bromothymol blue and bromophenol blue. These are readily extracted by chlorinated solvents or benzene for reading.[69] The indicator itself is insoluble in the organic solvent and does not form extractable salts with primary, secondary, and tertiary amines, or with alkaloids. For the reaction it is only essential that one alkyl radical be at least 4 carbons or longer, or is $C_6H_5CH_2$-. These include the methobromide of procaine and procaine amide, Banthine, the N-diethylmethylethanolamine bromide esters of 1-benzoylcyclopropanecarboxylic acid, of 2,2-diphenylpenten-4-oic, of α-phenoxycinnanic acid, and of 2-(*o*-chlorophenyl)-5,6-diydro-4H-pyran-3-carboxylic acid, the N-(2-hydroxyethyl-N-methylpiperidinium bromide ester of 1-benzoylcyclopropanecarboxylic acid, the 1-hydroxy-2-(diethylmethylamine)cyclohexane bromide ester of 2,2-diphenylpen ten-4-oic, and 4-benzoyl-4-carbethoxy-1,1dimethylpiperidinium bromide.

Modifications of the method make it applicable also to acetylcholine, hexamethonium, *d*-tubocurarine, neostigmine, and tetraethylammonium. Tertiary amines do not interfere. The lower limit of sensitivity is about 0.001 mg. per sample. Recovery of known quantities from dilute homogenates of liver, kidney, and heart was 100 ± 2 per cent.

By use of a carefully measured amount of indicator the colored complex may be read directly without extraction.[70] By this technic accuracy is to ±2 per cent in the range 100-500 ppm., ±5 per cent in range 50-100 ppm. By precipitation of Eriochrome Azurol B with an alkaline earth in the presence of a dispersing agent a fresh dispersion of the lake is obtained. Addition of a cation-active agent alters that

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[69] M. E. Auerbach, *Ind. Eng. Chem., Anal Ed.* **15**, 492-3 (1943); *Ibid.* **16**, 739 (1944); "Official and Tentative Methods of the Association of Official Agricultural Chemists," 7th Ed., pp. 464-6 Association of Official Agricultural Chemists, Washington D. C. (1950); Jean Pien, J. M. Desirant, and Mme. Rochelle, *Ann. fals. fraudes* **44**, 290-7 (1951); Ruth Mitchell and Byron B. Clark, *Proc. Soc. Exptl. Biol. Med.* **81**, 105-9 (1952).

[70] E. L. Colichman, *Anal. Chem.* **19**, 430-1 (1947).

at once.[71] A quaternary ammonium salt on a solid base such as paper or cloth is reacted with dilute potassium iodide solution to form a colored triiodide which is extracted with ethanol.[72]

By oxidation with potassium permanganate some quaternary ammonium compounds are converted to a chloroform-soluble form, giving a violet red.[73] This contains manganese, which is converted to permanganate and read in aqueous solution.

Samples—*Bottled beverages containing fruit juices.* Mix thoroughly and measure out 50 ml. of sample. Filter on a Büchner funnel and dilute the filtrate to 100 ml. with water. Extract the filter paper with small portions of ethanol until no more color is extracted. Transfer the alcoholic extract to a distilling flask, and add 10 mg. of bromophenol blue, 2 ml. of 1:1 hydrochloric acid, and 100 ml. of water. Steam-distil and collect a volume of distillate at least 100 ml. greater than the volume of ethanol in the extract. Cool the residue in the distilling flask, wash with 40, 30, and 30-ml. portions of petroleum ether, and proceed to develop with bromophenol blue on both the distillate and the extract from the residue.

Beer. Place 100 ml. of decarbonated beer in a steam-distilling flask and add 10 mg. of bromophenol blue and 2 ml. of 1:1 hydrochloric acid. Steam-distil and collect about 200 ml. of distillate. Cool the residue and wash with 100 and 50-ml. portions of petroleum ether. Develop with bromophenol blue.

Table sirup. Dilute 20 grams of sample to 100 ml. with water. Add 5 ml. of 0.04 per cent bromophenol blue solution and 1 ml. of 1:1 hydrochloric acid to an aliquot. Develop with bromophenol blue.

Eggs. Mix 12.5 ± 0.25 gram of a representative sample with 10 ml. of water. Transfer to a 250-ml. volumetric flask, using 5-10 ml. more of water. While twirling the flask, gradually add acetone, a little at a time, mixing constantly, until the flask is filled to the mark. Stopper and invert several times. After 10-15 minutes, filter, collecting 200 ml. of filtrate. Transfer to a 1-liter separatory funnel using 25 ml. of acetone for the transfer. Add 250 ml. of water and 25 ml. of 1:1 hydrochloric acid and mix. Extract successively with 300, 250, 150, and 100 ml. of petroleum ether, shaking gently to avoid formation of emul-

[71] John A. Hart and Edward W. Lee, *Tappi* 34, 77-9 (1951).

[72] O. B. Hager, E. M. Young, T. L. Flanagan and H. B. Walker, *Ind. Eng. Chem., Anal. Ed.* 19, 885-8 (1947).

[73] E. Flotow, *Pharm. Zentralhalle* 83, 181-5 (1942).

sions. Evaporate the extract to 50-75 ml. Add 10 mg. of bromophenol blue solution and use the chlorinated solvent called for under the procedure to complete the transfer.

Milk. Mix 20 ml. of milk, 50 ml. of water, and 20 ml. of 1 per cent acetic acid. Boil for 5 minutes and filter after several hours. Wash the residue with six successive 10-ml. portions of water and concentrate the filtrate to 1-2 ml. Add a few ml. of water and 5 ml. of 10 per cent sodium carbonate solution. Filter after a few minutes and wash the precipitate. Add 1 ml. of 0.04 per cent solution of bromophenol blue in 0.004 per cent sodium hydroxide solution and develop by bromophenol blue.

Serum or plasma. Precipitate the proteins by adding an equal volume of 6 per cent metaphosphoric acid, let stand 10 minutes, and centrifuge. To 3-4 ml. of supernatant liquid add 1.5 grams of sodium carbonate and 0.2 gram of dipotassium phosphate. Develop by bromophenol blue.

Urine. If the amount of quaternary ammonium compound is too small to permit dilution, modifications are introduced in accordance with Table 5. Certain compounds in other media also require modifica- of the method as shown in the table.

Procedure—*By bromophenol blue.* The reagent for color development will have been added in preparation of the sample by some technics. If alkali has not already been added, add 1 ml. of 20 per cent sodium carbonate solution to 2 ml. of sample to bring to pH 9. Prepare fresh daily a reagent solution containing 40 mg. of bromophenol blue in 50 ml. of a 30 per cent dipotassium phosphate solution. Add 1 ml. of this bromophenol blue preparation to the alkaline sample if indicator is not already present. As solvent, wash ethylene dichloride with 0.2 volume of 4 per cent sodium hydroxide solution, then 0.2 volume of 1:15 hydrochloric acid, followed by washing 3 times with water. Wash isoamyl alcohol in the same way and add 3 per cent to the washed ethylene dichloride. The solvent should be prepared each week.

Transfer the sample to a separatory funnel. Add an equal volume of ethylene chloride and shake 3-4 minutes. If the aqueous layer shows no excess of dye, add more before completing the extraction. Let stand until clear, draw off lower layer into another separatory funnel containing 10 ml. of 1 per cent sodium carbonate solution, and shake for 3-4 minutes. If the intensity of color of the lower layer is suitable, draw off the solvent, dry for a half-hour with anhydrous sodium

TABLE 5. EXAMPLES OF MODIFIED PROCEDURES SUITABLE FOR COMPOUNDS NAMED IN THE MEDIA LISTED

Compound	*Medium from which extracted*	*Max aliquot of sample, ml.*	*Alkaline salt used, g.*	*Dye reagent, ml.*	*Total aqueous phase, ml.**	*% iso-amyl alcohol in EDC*	*Limit of sensitivity μg/sample*
Hexamethonium	Urine (undiluted)	1	$1.2K_2HPO_4$ $0.6Na_2CO_3$	1	5	1.5	2.5
Acetylcholine†‡	Ringer's sol. & nerve tiss.	0.5	$0.3K_2HPO_4$ $0.3Na_2CO_3$	0.5	1	4	1
Tetraethylammonium	Ringer's solution	0.5–1	$0.3K_2HPO_4$ $0.3Na_2CO_3$	0.5	1–1.5	4	1.5
	Urine (undiluted)	0.5	$0.4Na_2CO_3$	0.5	1	4	2.5
	Serum (diluted 1:10)	1	$0.8Na_2CO_3$	0.5	1.5	4	2.5
Neostigmine	Ringer's solution	1	$0.4Na_2CO_3$	0.5	1.5	4	2.5
	Urine (undiluted)	1	$0.8Na_2CO_3$	0.5	1.5	4	2.5
d-Tubocurarine	Ringer's solution	1	$0.4Na_2CO_3$	0.5	1.5	4	2.5
	Urine (undiluted)	1	$0.8Na_2CO_3$	0.5	1.5	4	2.5

* Where aqueous phase is greater than sum of sample and dye, distilled water was added.
† Period of shaking limited to 10 min. ‡ Lower limit of sensitivity for choline is 15 μg/sample.

sulfate, and read at 610 mμ. If too deep, add more ethylene chloride before separation.

For estimation without extraction dilute the sample containing up to 25 mg. of the quaternary ammonium compound with 1 ml. of 10 per cent sodium carbonate solution and exactly 2 ml. of 0.040 per cent bromophenol blue solution. Read after exactly 5 minutes around 645 mμ against a blank from which the dye was omitted. In the calibration curve the color of the diluted dye corresponds to absence of the quaternary compound.

By eriochrome Azurol B. As reagent dissolve 0.2 gram of the dye and 13.5 grams of potassium carbonate in water and dilute to a liter. Mix 10 ml. of this solution with 10 ml. of 1.25 per cent magnesium sulfate in 0.5 per cent aqueous polyvinyl alcohol to precipitate the fresh lake. At once add 25 ml. of the sample solution. Read against a reagent blank.

As the triiodide. Textiles and paper. Starch must be absent. The method was developed for octadecyldimethylbenzylammonium chloride. Immerse a 0.6-gram piece of fabric or paper containing quaternary ammonium salts, in a mixture of 25 ml. of water and 1 ml. of 2 per cent potassium triiodide solution. Stir for about 20 minutes and rinse with 10-ml. portions of 5 per cent potassium iodide solution until the rinse solution is colorless. Usually 3 portions will be required. The indefinite polyiodide has been reduced to the fairly stable triiodide. Standing or excessive rinsing will give low results. Squeeze out as much of the rinse solution as possible.

Extract the triiodide by immersing the sample in 20 ml. of ethanol and working with that solvent. Filter through dry inorganic filter and read at 450 mμ against an ethanol blank.

By oxidation with potassium permanganate. Acidify 2 ml. of the solution with sulfuric acid and add 1 ml. of chloroform. Add concentrated potassium permanganate solution titrametrically until the color of excess persists in the aqueous layer. Separate the chloroform layer quantitatively and evaporate to dryness. Add 1 ml. of concentrated nitric acid to the residue and evaporate to dryness. Repeat that operation. Take up the residue in 2 ml. of water and add a drop of 2 per cent silver nitrate solution. Heat to boiling for 10 minutes with 0.5 gram of moist potassium persulfate (Vol. II, page 394) to destroy organic matter and oxidize manganese to permanganate. Dilute to a volume which gives an appropriate intensity of color, read at

525 mμ, and compare with a calibration curve prepared with the same quaternary ammonium compound.

Trimethylethanol Ammonium Hydroxide, Choline

Choline in vegetable phosphatides is determined by hydrolysis and precipitation of the liberated choline as the reineckate, the precipitate being redissolved and read.[74] By this technic accuracy to ±2 per cent is obtainable over the range 0.64-15.5 mg. Precipitation must not be from a solution containing more than 5 mg. per ml. or occlusion of the reagent will cause unduly high results. Acetyl choline also reacts, preferably after hydrolysis.

An indirect method is based on the chromium content of the precipitate.[75] By it the reineckate precipitate is oxidized with alkaline hydrogen peroxide and the color developed with diphenylcarbazide. Choline is precipitated by an aqueous solution of hexanitrodiphenylamine and magnesium oxide and read in acetone.[76] The same reaction is applicable to acetylcholine, carbamoylcholine, and succinyldicholine.

Sample—*Lecithin. Acid hydrolysis.* Hydrolyze a 0.2-gram sample by refluxing for 15 hours with 20 ml. of 1:2 hydrochloric acid. Cool and extract fatty acids with 10, 10, and 5 ml. of ether. Neutralize the aqueous layer by addition of 30 per cent sodium hydroxide solution and dilute to 25 ml. for the use of aliquots with the aqueous reagent.

Alkaline hydrolysis. Heat a 200-mg. sample in 2 ml. of absolute methanol with 0.2 ml. of 30 per cent potassium hydroxide solution in a sealed tube at 100° for 1 hour. Cool to the same temperature at which the previous volume was measured and take a 2-ml. aliquot of the tube contents. Add 1:35 sulfuric acid until neutral to bromocresol and evaporate to dryness at not over 50°. Take up the soluble matter and transfer the insoluble portion with three successive 3-ml. portions of benzene. Centrifuge and discard the benzene solution. Dry the residue for 2 hours at not over 50°. Extract the residue with three

[74] Florence J. R. Beattie, *Biochem. J.* **30**, 1554-9 (1936); M. H. Thornton and F. K. Broome, *Ind. Eng. Chem., Anal Ed.* **14**, 39-41 (1942); R. W. Engel, *J. Biol. Chem.* **144**, 701-10 (1942); C. Entenman, Alvin Taurog, and I. L. Chaikoff, *Ibid.* **155**, 13-18 (1944); Richard J. Winzler and Emily R. Meserve, *Ibid.* **159**, 395-7 (1945); Paul Fleury, *Compt. rend.* **226**, 441-2 (1948); Paul Fleury and Hubert Guitard, *Ann. pharm. franc.* **6**, 272-4 (1948).

[75] A. D. Marenzi and C. E. Cardini, *J. Biol. Chem.* **147**, 363-70 (1943).

[76] Gunnar Samuelsson, *J. Pharm. and Pharmacol.* **5**, 239-44 (1953).

successive 3-ml. portions of hot absolute ethanol. Combine the extracts, dilute to 10 ml., centrifuge to separate any suspended matter, and use an aliquot of the clear solution for determination by the methanolic reagent.

Tissue. Extract the tissue with alcohol and ether and concentrate the extract to low volume under reduced pressure in an atmosphere of carbon dioxide. Redissolve the lipids in petroleum ether. Concentrate an aliquot of this to low volume and precipitate phospholipids with acetone and magnesium chloride.

Centrifuge, dissolve the precipitate in a mixture of methanol and ether, and transfer to an apparatus for hydrolysis of phospholipids. The apparatus consists of a 125-ml. conical flask having a side-arm of 2-ml. capacity set into the flask at an angle of 45°, about 3 cm. from the top. Evaporate the methanol-ether solution to about 5 ml. and add 15 ml. of saturated barium hydroxide solution. Place on a steam bath for 2 hours and shake frequently. Bring almost to dryness and acidify with 1.7 ml. of 1:1 hydrochloric acid, heating and shaking to complete acidification. Add 15 ml. of petroleum ether and heat on the steam bath with shaking. As the bubbling ceases, pour off the petroleum ether phase, and retain the aqueous phase in the side-arm. Extract twice more with 15 ml. of petroleum ether. This procedure extracts fatty acids and the extract may be discarded. Dissolve the salt in the flask by adding 5-8 drops of water and heating on the steam bath. Transfer with two 2-ml. portions of 1:9 hydrochloric acid for development with diphenylcarbizide.

Plasma.[77] Boil 0.5 ml. of heparinized plasma with 8 ml. of 1:1 ethanol-acetone and filter. Use 5 ml. and 5 ml. of solvent to wash the flask and filter. Concentrate the extract and washings to about 0.5 ml. and add 10 ml. of saturated barium hydroxide solution. Heat for 2 hours in boiling water, cool, add thymolphthalein indicator, and neutralize with acetic acid to pH 8.9. Filter and wash the filter with 3 ml. and 3 ml. of water. Complete with the aqueous reineckate reagent.

Procedure—*By aqueous reineckate reagent.* Prepare a saturated solution of ammonium reineckate, $NH_4[Cr(NH_3)_2(SCN)_4]H_2O$, before use each time by stirring a quantity of the salt in water for 15 minutes and then filtering. Approximately 1 ml. of this solution will precipitate about 10 mg. of choline.

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[77] V. Posborg Petersen, *Scand. J. Clin. Lab. Invest.* 2, 14-20 (1950).

Add slowly and with stirring, 5 ml. of the reagent and allow to stand for 30 minutes with frequent agitation. Centrifuge for 10 minutes and discard the supernatant liquid. Wipe the mouth of the tube to remove excess reineckate. Wash down the sides of the tube with 3 ml. of 1:9 hydrochloric acid and agitate to wash the precipitate. Avoid prolonged agitation as it causes the precipitate to dissolve. Centrifuge again for 10 minutes. Again discard the liquid and wipe the tube. Dissolve the reineckate in 10 ml. of acetone and centrifuge for 5 minutes. Read at 500-550 mμ against acetone. For small amounts read in the ultraviolet at 327 mμ for greater sensitivity.

By methanolic reineckate reagent. Transfer a 5-ml. aliquot of the sample in absolute ethanol. Adjust to approximately pH 4 and add a few drops of a 2 per cent solution of Reinecke's salt in absolute methanol. Choline is precipitated. Stir for 2 minutes, refrigerate for an hour, and centrifuge. Decant and wash the residue with 3 ml. of methanol. Wash with 3 ml. of ether, then dry for 30 minutes at 37°. Take up the residue in 5 ml. of acetone and read in the range 520-570 mμ against a reagent blank.

By diphenylcarbazide. The reagent is a solution of 0.2 per cent diphenylcarbazide in ethanol. It has a faint rose color which darkens after a few days standing, but it can be used nevertheless.

Render an aqueous solution containing 0.015-0.1 mg. of choline slightly acid with 1:9 hydrochloric acid and add an equal volume of saturated aqueous ammonium reineckate. Cool in ice water for at least 20 minutes to complete precipitation of choline. Centrifuge for 4 minutes. Chill the tube carriers beforehand to avoid solution of the precipitate. Remove the supernatant liquid and wash the precipitate with 0.5 ml. of ice-cold ethanol. Chill a few minutes, then repeat the washing and centrifuging. Dissolve the precipitate in 1 ml. of acetone and transfer with 2-3 ml. of 60 per cent acetone. Add 2 ml. of water, 0.2 ml. of 10 per cent sodium hydroxide solution, and 0.1 ml. of 30 per cent hydrogen peroxide for each 0.05 mg. of choline in the sample.

Heat the tube carefully in boiling water as rapid evaporation of acetone induces spattering. In order to decompose the hydrogen peroxide, allow to remain in the bath 20-30 minutes after all the acetone has evaporated. During the heating a yellow color forms due to chromate. Occasionally this color deepens, an indication that there is not enough hydrogen peroxide present. In this case, add 0.1 ml. more of 30 per cent hydrogen peroxide and heat.

After oxidation of chromium is completed, cool, dilute with 3 ml. of water, and add 2 ml. of 1:9 sulfuric acid and enough 0.25 per cent ethanolic diphenylcarbazide solution to develop the full color intensity. A violet-red color will form. Dilute to 25 ml. with water and read at 530 mμ against a blank consisting of 2 ml. of 1:9 sulfuric acid and 2 ml. of the diphenylcarbazide solution diluted to 25 ml. with water.

By hexanitrodiphenylamine. As reagent mix 12 grams of hexanitrodiphenylamine with 5 grams of magnesium oxide and 400 ml. of water. After 20 hours, filter. If it becomes turbid before use, filter again.

To an ice-cold solution of 0.2-0.4 mg. of choline add 1 ml. of reagent. Chill in ice for 30 minutes and filter. Wash with a saturated aqueous solution of the precipitate and dry. Take up the precipitate in acetone, dilute to 100 ml., and read at 415 mμ against acetone.

Acetylcholine

Acetylcholine is rapidly converted in stoichiometric proportions to hydroxamic acid by hydroxylamine in alkali.[78] This is determinable by ferric chloride in acid solution. Protein in the sample will be precipitated upon addition of the acid ion solution and is removed by filtration or centrifugation. Such precipitation is hastened by addition of trichloroacetic acid. Phospate, sulfate, fluoride, and oxalate are iron-binding anions and inhibit the reaction.[79] If a suitable excess of iron is present, the effect of phosphate is suppressed completely; sulfate and borate exhibit little interference. A reaction with hexanitrodiphenylamine described under choline above is also applicable.

Procedure—Prepare the reagent by mixing equal volumes of 16 per cent hydroxylamine hydrochloride and 14 per cent sodium hydroxide solutions. This will keep for about 3 hours at room temperature. Mix 2 ml. of the reagent with 0.1 ml. of the sample solution. After about 1 minute adjust the pH to 1.2 ± 0.2 with 1 ml. of concentrated hydrochloric acid. Add 1 ml. of 10 per cent ferric chloride hexahydrate in 1:120 hydrochloric acid. Read the purple-brown color immediately at 540 mμ. As the blank repeat the procedure as described but reverse the order of addition of hydroxylamine, alkali, and acid.

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78 Shlomo Hestrin, *J. Biol. Chem.* **180**, 249-61 (1949).
79 F. Lipmann and L. C. Tuttle, *Ibid.* **159**, 21 (1945).

Carbamoylcholine

A reaction with hexanitrodiphenylamine described under choline (page 71) is applicable.

Succinyldicholine

A reaction with hexanitrodiphenylamine described under choline (page 71) is applicable.

β-Dimethylaminoethyl Benzhydryl Ether, Benadryl

As a general reaction, organic bases react with methyl orange to form colored complex salts which are soluble in appropriate organic solvents.[80] The determination of β-dimethylaminoethyl benzhydryl ether, having the trade name of Benadryl, is based on this.[81] Methyl orange enters the organic phase in direct proportion to the concentration of the organic base with which it is combined.

A double extraction technic to avoid interferences consists of extracting the Benadryl with heptane from alkaline solution, extracting the heptane with dilute hydrochloric acid to get the organic base back into the aqueous phase as its salt, and then re-extracting into ethylene dichloride. Benzophenone and benzohydrol give no color by the methyl orange reaction.

Samples—*Plasma.* Add 4 ml. of 0.4 per cent sodium hydroxide solution to 3 ml. of oxalated plasma containing 0.001-0.01 mg. of Benadryl per ml. Shake this with 25 ml. of heptane for 10 minutes and centrifuge. Shake 20 ml. of this solution with 6 ml. of 1:120 hydrochloric acid for 5 minutes. The Benadryl will be transferred to the acid layer. Pipet off 5 ml. of the acid layer and add to it 1 ml. of 4 per cent sodium hydroxide solution and 10 ml. of ethylene dichloride. Shake for 5 minutes to transfer the Benadryl to the organic phase. Remove the aqueous layer and use the ethylene dichloride layer or an aliquot as sample.

Tissue. Use a motor-driven apparatus with a stainless steel rotor to homogenize a 1-gram sample of tissue containing about 0.02 mg. of

[80] Bernard B. Brodie and Sidney Udenfriend, *J. Biol. Chem.* 158, 705-14 (1945).
[81] E. Philip Gelvin, Thomas H. McGavack and I. J. Drekter, *Bull. New York Med. Coll.* 9, 51-5 (1946); Wesley A. Dill and Anthony J. Glazko, *J. Biol. Chem.* 179, 395-401 (1949).

Benadryl. Add water during the homogenizing process so that the final homogenate is about 5 ml. Dilution is a critical point in the procedure; excessive dilution will cause a tendency to form emulsions with heptane and insufficient dilution makes complete extraction difficult. Transfer the homogenate to a bottle, add 5 ml. of 0.4 per cent sodium hydroxide solution and 25 ml, of heptane, and shake for 15 minutes. After separation shake 20 ml. of the heptane layer with 6 ml. of 1:120 hydrochloric acid for 5 minutes. Add 1 ml. of 4 per cent sodium hydroxide solution to 5 ml. of the acid and shake with 10 ml. of ethylene dichloride. Centrifuge and take an aliquot of the ethylene dichloride layer for color development.

Urine. Although urine contains a number of organic bases which cause interference, an appropriate buffer reduces the effect. Prepare the buffer by dissolving 12.4 grams of boric acid and 14.9 grams of potassium chloride in 800 ml. of water. Adjust the pH to 8 with the glass electrode by addition of 4 per cent sodium hydroxide solution, and dilute to 1 liter.

Make a 10-ml. urine sample alkaline with 4 per cent sodium hydroxide solution and extract with 25 ml. of heptane for 10 minutes. Centrifuge and then shake 20 ml. of the heptane solution with an equal volume of the buffer solution. Discard this buffer and extract 20 ml. of the pre-extracted heptane layer with 6 ml. of 1:120 hydrochloric acid. Mix 5 ml. of this acid layer with 1 ml. of 4 per cent sodium hydroxide solution and 10 ml. of ethylene dichloride. Shake for 5 minutes to transfer the Benadryl to the organic phase. Separate the ethylene dichloride layer as the sample solution.

Procedure—Saturate a 3.1 per cent boric acid solution with methyl orange by shaking mechanically overnight. Filter off undissolved methyl orange, wash the solution three times with ethylene dichloride, and store in a bottle containing a layer of ethylene dichloride. Add 0.5 ml. of the methyl orange reagent to 10 ml. of the ethylene dichloride solution which contains up to 0.003 mg. of Benadryl and shake mechanically for 5 minutes. Remove as much as possible of the methyl orange layer and centrifuge the ethylene dichloride layer for about 10 minutes. Add 5 ml. of the ethylene dichloride layer to 0.5 ml. of a solution made up of 2 ml. of concentrated sulfuric acid and 98 ml. of absolute ethanol. Be sure not to transfer any methyl orange reagent at this point. Mix thoroughly and read at 535 mμ against a reagent blank.

Saccharin

One method of estimation of saccharin is by hydrolysis to ammonia and subsequent estimation by Nessler's reagent.[82] Another is by fusion with phenolsulfonic acid to form phenolsulfonphthalein, phenol red.[83] In general, in beverages containing less than 0.005 gram per 100 ml. the results are high. The second method was developed for ice cream cones and adapted to soft drinks. Accuracy is a little better than ±10 per cent.

The pink-violet color developed with oxidized saccharin, copper sulfate, and sodium nitrite is also applicable.[84] Fruit juices interfere, but otherwise results by this method are also accurate to about ±10 per cent. A sample oxidized with potassium permanganate can be estimated by ferric ion.[85] Saccharin gives a violet precipitate with naphthylamine and copper sulfate which in chloroform solution is suitable for estimation.[86]

Sample—*Alcoholic liquids.* Heat 100 ml. of sample on a water bath to remove ethanol. Usually evaporation to slightly less than half volume will accomplish this. If the solution is sirupy, dilute with an equal volume of water before evaporating. When removal of ethanol is complete, dilute to 50 ml. and add 3 ml. of glacial acetic acid. Mix and add a slight excess of 20 per cent neutral lead acetate solution. Mix well, dilute to 100 ml., and mix. Filter, protecting from evaporation, and use an aliquot for development as phenol red.

Fruit juices and sirups. Dilute 50 ml. of sample to about 75 ml. with water and add 3 ml. of glacial acetic acid. Mix and add a slight excess of 20 per cent neutral lead acetate solution. Mix well, dilute to

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[82] Guiseppe Testoni, *Z. Nahr. Genussm.* **18**, 577-87 (1909); A. F. Lerrigo and A. L. Williams, *Analyst* **52**, 375-83 (1927); John C. Krantz, Jr., *J. Assoc. Official Agr. Chem.* **17**, 193-95 (1934); Ibid. **18**, 372-3 (1935); Ibid. **19**, 205-6 (1936); "Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists," 7th ed., pp. 467-8, Association of Official Agricultural Chemists, Washington, D. C. (1950).

[83] L. J. Cross and J. L. Perlman, *N. Y. State Dept. Agr. and Markets, Ann. Report* **1930**, 89-90; Wm. F. Reindollar, *J. Assoc. Official Agr. Chem.* **24**, 326-7 (1941); E. G. Whittle, *Analyst* **69**, 45-7 (1944).

[84] V. A. Rozanova, *Obshchestvennoe Pitanie* **9**, No. 1, 19-25 (1941); P. A. Soifer, *Gigiena i Sanit.* **11**, No. 6, 33-6 (1946).

[85] Jacques Lavagne, *Ann. pharm. franc.* **3**, 26-9 (1945).

[86] A. Gandini, *Farmaco sci. e tec. (Pavia)* **1**, 34-8 (1946).

100 ml., and mix. Filter, protecting the filtrate from evaporation, and use an aliquot for development as phenol red.

Solid or semi-solid products. Add boiling water to a 50-gram sample to dilute to 75 ml. Let stand for 2 hours, shaking occasionally. Add 3 ml. of glacial acetic acid and mix thoroughly. Add a slight excess of 20 per cent neutral lead acetate solution. Dilute to 100 ml. with cold water and mix. Let stand for 20 minutes, filter, protecting from evaporation, and use an aliquot for development as phenol red.

Procedure—*By Nessler's reagent.* Add 2 ml. of concentrated hydrochloric acid to 50 ml. of sample and extract with two successive 50-ml. portions of ether. Filter the combined ether extracts through cotton and wash with 5 ml. of distilled water acidified with a drop of concentrated hydrochloric acid. Evaporate the ether solution to dryness on a water bath. Add 5 ml. of water and 6 ml. of concentrated hydrochloric acid to the residue. Evaporate to about 1 ml. Dilute to 50 ml. with ammonia-free water. Add 2.5 ml. of Nessler's reagent (page 181) and compare with a standard (Vol. II, pages 816-817) similarly treated or read the transmittance at 420 mμ against a reagent blank. The color is stable for about 30 minutes.

As phenol red. Extract a measured volume of alcohol-free sample containing 0.005-0.03 gram of saccharin with ether. Evaporate the ether extract to dryness. Fuse the residue from ether extraction with a suitable amount of phenolsulfonic acid for 2 hours at 130-140°. Let cool and dissolve in water. Make alkaline and dilute to a known volume. Read at 480 mμ against a reagent blank.

By copper sulfate and sodium nitrite. To 10 ml. of neutral, ethanol-free sample, add a drop of 1:3 sulfuric acid and extract with 3 successive 5-ml. portions of ether. Combine the ether extracts and evapor [illegible] until ethanol- and ether-free. Take up by heating with water [illegible] and dilute to 15 ml. to contain not less than 0.02 per cent of s[illegible] Add 0.5 ml. of 0.5 per cent hydrogen peroxide. Add 0.[illegible] reagent containing 0.3 per cent of copper sulfate pentah[illegible] acetic acid, then 0.5 ml of 2 per cent sodium nitrite [illegible] to 20 ml. and read after 20 minutes against a reag[illegible]

By ferric ion. Extract a sample containing [illegible] successively with three 5-ml. portions of ethe[illegible] extracts to dryness on a water bath and t[illegible] of 1:360 sulfuric acid. Heat in a water [illegible]

sium permanganate dropwise until excess is present for 2 minutes. Decolorize with a drop or two of 10 per cent ethanol. Filter and wash the filter to give 100 ml. of solution. Cool, extract with three 10-ml. portions of ether, and filter the combined extracts through a dry paper. Evaporate the ether and take up the residue with three 3-ml. portions of boiling water. Cool and add 2 drops of 5 per cent solution of ferric chloride hexahydrate and 2 drops of 6 per cent hydrogen peroxide. After 5 hours read against a reagent blank.

Acriflavine

Acriflavine is a mixture of 2,8-diamino-10-methylacridinium chloride and 2,8-diaminoacridine. Both give a purple to burgundy-red color with nitric acid containing oxides of nitrogen or with sodium nitrite and hydrochloric acid.[87]

Procedure—Add 1 ml. of 10 per cent nitric acid, freshly prepared from commercial fuming acid, to 1 ml. of sample containing about 0.1-0.3 mg. of test substance. Stir vigorously until a maximum color is developed. Dilute to 10 ml. and read against a reagent blank.

m-Benzaminosemicarbazide, Cryogenin

Cryogenin gives an orange color with concentrated hydrochloric acid which is more intense if some chloric acid is present.[88]

Procedure—To 1 ml. of sample solution containing 1-10 mg. of cryogenin add 1 drop of a 0.5 per cent solution of potassium chlorate. Add 1 ml. of concentrated hydrochloric acid and mix. After 30 ...tes read against a reagent blank.

[82] ...
A. L. Wi...
Agr. Chem. ...

Suramin

"Official and T... ...eveloped by suramin with 2-*p*-dimethylaminostyryl-
tural Chemists," 7th ... ...e methochloride is suitable for its estimation.[89]
Washington, D. C. (19...
[83] L. J. Cross and J. ...lutions, it is applicable to serum or plasma without
Report 1930, 89-90; Wm. F. ...
(1941); E. G. Whittle, *Analys*...
[84] V. A. Rozanova, *Obshchestv*... ...*ng. Chem., Anal. Ed.* 2, 114 (1930); M. J.
Gigiena i Sanit. 11, No. 6, 33-6 (194... ...809-11 (1930).
[85] Jacques Lavagne, *Ann. pharm. fr*... ...*Bordeaux* 68, 51-2 (1930).
[86] A. Gandini, *Farmaco sci. e tec. (Pavi*... ...*t, Biochem. J.* 42, 574-7 (1948).

Procedure—Add 0.2 ml. of sample to 2 ml. of 0.7 per cent sodium chloride solution. Add 5 ml. of 0.001 per cent aqueous 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride. Dilute to 10 ml. and read against a reagent blank.

4,4′-Diamidostilbene, Stilbamidine

The brilliant blue fluorescence of stilbamidine in ultraviolet light is suitable for photometric estimation after separation from interfering substances.[90] All aqueous solutions must be handled in dim artificial light. Ethanolic solutions have 50 per cent more fluorescence than an equivalent aqueous solution. The fluorescence deteriorates rapidly during measurement. Readings conform to Beer's law over the range 0.001-0.15 mg. per ml. Accuracy is to ±2 per cent and results tend to be low. Proteins in the sample do not interfere. A general reaction of aromatic amidenes by condensation with glyoxal (page 231) is also applicable.

Samples—As a sorption column use 25 cm. of 9-mm. tubing closed to 0.5 mm. at the bottom. A funnel top is always desirable. Wash 50-100 mesh Decalso with 3 per cent acetic acid, then with distilled water, and dry. Place 1 cm. of moistened sand in the bottom of the column and on it 6 cm. of the processed Decalso. Tap to obtain uniform distribution.

Urine or plasma. Allow 5 ml. of sample containing 0.001-0.03 mg. of stilbamidine to percolate through the column under the influence of gravity. Efficiency can be checked by brief exposure to a weak source of ultraviolet light. Wash the column with 60 ml. of water, applying gentle suction. Elute under gravity with three successive 4-ml. portions of a 1:1 mixture of 1:25 hydrochloric acid and ethanol.

Tissue. Grind 1-2 grams of tissue in a mortar with an equal amount of silica sand. Wash into a flask with 30-100 ml. of 1 per cent acetic acid per gram of tissue, depending on the concentration of stilbamidine in the sample. Shake for 20 minutes to extract the diamidine and then centrifuge. Decant the supernatant liquid and centrifuge again.

Pour 5-10 ml. of clear centrifugate onto the column. Wash under gentle suction 4 times with 20-ml. portions of water. Elute the diamidine with two 4-ml. portions and one 3-ml. portion of a 1:1 mixture of 1:25 hydrochloric acid and ethanol.

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[90] Abraham Saltzman, *J. Biol. Chem.* **168**, 6[illegible]3 (1947); F. Lieben and I. Snapper, *Exptl. Med. Surg.* **8**, 357-60 (1950).

Procedure—Add 1 ml. of concentrated hydrochloric acid to the combined eluates, dilute to 25 ml., and mix. Exactly 15 minutes later start exposure in the cell. Use the usual primary and secondary vitamin B_1 filters and cut down the intensity of the beam about 70 per cent by interposing a wire screen. Instrument settings are 50 for 0.001 mg. of quinine sulfate in 1:360 sulfuric acid and zero for the blank. Exactly one minute after exposure is started, read and compare with a curve prepared from stilbamidine isethionate.

2-Hydroxystilbamidine

For the estimation of 2-hydroxystilbamidine prepare the samples exactly as for stilbamidine but develop the yellow fluorescence with sodium hydroxide.[91]

Procedure—Add 40 per cent sodium hydroxide to the eluate until neutralized and a maximum yellow fluorescence is developed and dilute to 25 ml. Read against a reagent blank.

Dihydroxystilbamidine

When extracted, dihydroxystilbamidine gives a yellow fluorescence in alkaline solution.[92]

Samples—*Tissue.* Elute as for stilbamidine. Then to the combined eluates add 0.4 ml. of 2 per cent glyoxal solution, followed by 0.4 ml. of 2 per cent sodium bisulfite solution. Add 0.5 ml. of 8 per cent benzaldehyde in ethanol and use as sample.

Urine or plasma. Prepare exactly as for stilbamidine.

fo **Procedure**—Add 1 ml. of 40 per cent sodium hydroxide solution
temp development of a yellow fluorescence. After 10 minutes at room
Dilute erature with frequent shaking, add 10 ml. of 9:1 ethanol-ether.
filter with 25 ml. with water and read the fluorescence. Use a primary
maximum at 370 mμ and an orange-yellow secondary filter.

Isod

canoic Acid Vanillyl Amide, Capsaicin

Capsaicin is the
important constituent o pungent oleoresin principle which is the most
f red pepper. It is the vanillyl amide of iso-

[91] F. Lieben and I. Snapper, Exptl. Med. Surg. 8, 357-60 (1950).
[92] Loc. cit.

lecanoic acid, usually classified as an alkaloid. It reacts with vanadium oxychloride to give a blue color in anhydrous acetone, ether, or chloroform.[93] Extracts made with carbon tetrachloride are inferior. There is interference by other phenols.

It is also satisfactory to use typical phenol reagents such as phosphotungstic-phosphomolybdic acid[94] or phosphomolybdic acid.[95] A vanillin standard is convenient.

Sample—*Capsicum.* Dry a quantity of powdered paprika for 24 hours over calcium chloride. Add 100 ml. of ether to a 2-gram sample and shake occasionally over a 1-hour period. Remove a 50-ml. aliquot of the clear upper layer and evaporate to dryness on a warm bath. Dissolve the oleoresin in 10 ml. of petroleum ether and extract it with five 10-ml. portions of 60 per cent methanol. Discard the petroleum ether layer. If the combined extracts have a considerable amount of color, shake further with petroleum ether. Evaporate the methanolic solution almost to dryness and place in a desiccator for 24 hours. Dissolve the residue in 50 ml. of dry acetone and use an aliquot for development with vanadium oxychloride.

Oleoresin. Dissolve 1 gram of oleoresin in 20 ml. of purified and deodorized kerosene. Add 20 ml. of 60 per cent acetone containing 1.25 per cent of sodium chloride. Shake gently for about 5 minutes and let separate. Withdraw the cloudy layer and similarly extract the upper layer with 20, 20, and 20 ml. of the acetone solution of sodium chloride. Shake the combined extracts with 5 ml. of the kerosene. After 1 hour withdraw the lower layer and shake mechanically for a half-hour with 0.5 gram of Filter-cel. Dilute to 100 ml. with 60 per cent acetone and filter clear.

Reduce a 50-ml. aliquot to 20 ml. at not over 65°. The acetone is evaporated and the capsaicin separates as an oily sediment. Heat as little as possible. Cool and add 10 ml. of 2 per cent sodium hydroxide solution. This will dissolve the sediment on stirring. Use two 5-ml. portions of 2 per cent sodium hydroxide solution and two 5-ml. por-

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93 K. Von Fodor, *Z. Untersuch. Lebensm.* **61**, 94-100 (1931); Georges Deniges, *Bull. soc. pharm. Bordeaux* **70**, 182-3 (1932); Linwood F. Tice, *Am. J. Pharm.* **105**, 320-5 (1933); Alice Hayden and C. B. Jordan, *J. Am. Pharm. Assn.* **30**, 107-9 (1941); C. B. Jordan, E. W. Robel, and H. O. Thompson, *Bull. Natl. Form. Comm.* **10**, 49-57 (1942).

94 Horace North, *Anal. Chem.* **21**, 934-6 (1949).

95 J. Buchi and F. Hippenmeier, *Pharm. Acta. Helv.* **23**, 327-36.

tions of water for transfer to a separatory funnel. Add 5 grams of sodium bicarbonate and 150 ml. of petroleum ether and shake moderately for 15 minutes. Let the layers separate overnight and discard the lower layer. Filter the petroleum ether layer and wash the filter with petroleum ether. Carefully exclude from the filtrate a yellow substance which separates. Extract the petroleum ether solution with 10 ml. of 2 per cent sodium hydroxide solution and add 10 drops of ethanol to promote separation. Filter the lower layer and extract the residual ether with 10, 10, and 10 ml. of water, filtering these extracts. Dilute the filtrate to 50 ml. for development of aliquots by phosphotungstic-phosphomolybdic acid.

Spice. Extract 10 grams in a Soxhlet with acetone. Evaporate the acetone from the extract at under 60° and treat as an oleoresin sample.

Drugs. Dry the sample overnight in a desiccator. Mix 10 grams with anhydrous acetone and dilute to 100 ml. Shake occasionally and let sediment for development by vanadium oxychloride.

Procedure—*By vanadium oxychloride.* Carefully add freshly prepared 1 per cent vanadium oxychloride solution in carbon tetrachloride to an aliquot of the sample drop by drop, until no further deepening of color occurs. Excess reagent alters the color from blue to green. It is desirable to treat several tubes of samples with varying amounts of reagent and use that showing the maximum blue color but no green. The color fades appreciably within 15 minutes. Read against a sample blank.

By phosphotungstic-phosphomolybdic acid. Mix a 5-ml. aliquot of sample solution and 5 ml. of reagent (Vol. III, page 116). After 5 minutes dilute to 50 ml. with saturated sodium carbonate solution. Shake mechanically for 30 minutes to precipitate sodium phosphate completely. Filter and read at 610 mμ or 650 mμ. Interpret in terms of a vanillin standard and multiply the results by 2 to give capsaicin.

With phosphomolybdic acid. Dissolve a sample containing about 10 mg. of pure capsaicin in 45 ml. of 0.4 per cent sodium hydroxide solution by gentle warming on a water bath. Cool, dilute to 50 ml. with [illegible] per cent sodium hydroxide solution, and shake vigorously. Add [illegible]f this solution to 3 ml. of 3 per cent solution of phosphomolybdic [illegible]er 1 hour in daylight, read against a reagent blank.

impor

[91] F. Lieb

[92] Loc. cit.

THIAMINE, ANEURINE, VITAMIN B_1

Thiamine does not couple with diazotized amines in acid solution,[96] ut coupling does occur in alkaline solution, leading to various colors. he most stable and specific of these is diazotized *p*-aminoacetophenone hich forms a purple-red compound. While insoluble in water, this is xtracted into organic solvents.[97] In either aliphatic or aromatic lvents the color conforms to Beer's law. Accuracy to ±3 per cent is be expected.

The colored precipitate is insoluble in water, concentrated alkali, ilute acids, and petroleum ether. It is soluble in ethanol, propanol, utanol, isobutanol, acetone, dioxane, glacial acetic acid, ether, carbon etrachloride, chloroform, benzene, toluene, and xylene. Toluene and ylene are preferred for extraction as least soluble in water.

No colored precipitate is produced with acetic acid, acetone, adrenlin, adenine, albumin, ammonium nitrate, amidopurine, arginine, atroine, barbiturates, benzoates, benzylamine, betaine, bromides, caffein, asein hydrolyzate, choline, chlorobutanol, citrates, creatine, creatinine, ytosine, dextrose, ethanol, ethylamine, fructose, ferric ammonium itrate, galactose, gelatin hydrolyzate, glutathione, glycerophosphates, guanidine, guanine, hydrazine, hydroquinone, hydroxylamine, iodides, ysine, insulin, lactose, lactates, liver extract, methanol, nicotine, nicotinic acid, nicotinamide, nucleic acid, oxalic acid, pepsin, phenol, phloroglucinol, pilocarpine, quinine, resorcinol, riboflavin, saccharin, sarcosine, salicylates, sucrose, theelin, thioneine, thiochrome, trimethylamine, thymine, tyramine, uracil, urea, uric acid, vitamins A and D, or twenty-two amino acids. Inositol gives a greenish-blue precipitate but is separable by washing the thiamine precipitate with dilute acid. Histidine and histamine both give orange with the reagent, tryosine pink, adrenalin red changing to violet and yellow. None are extracted by xylene. Thymol blue is useful as a pH indicator, as it is not extracted with the color from the test substance.

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[96] Henry W. Kinnersley and Rudolf A. Peters, *Biochem. J.* **28**, 667-70 (1934).

[97] Harry J. Prebluda and E. V. McCollum, *Science* **84**, 488 (1936); *J. Biol. Chem.* **127**, 495-503 (1939); Daniel Melnick and Henry Field, Jr., *Ibid.* **127**, 505-40 (1939); *Ibid.* **130**, 97-107 (1939); *Proc. Soc. Exptl. Biol. and Med.* **39**, 317-19 (1938); R. A. Brown, Eva Hartzler, Gail Peacock, and A. D. Emmett, *Ind. Eng. Chem., Anal Ed.* **15**, 494-5 (1943); Melvin Hochberg and Daniel Melnick, *J. Biol. Chem.* **156**, 53-9 (1944); Carl M. Lyman, Robert Ory, Mary Trant, and Gene Rich, *Anal. Chem.* **24**, 1020-1 (1952).

Inactive degradation products of thiamine do not react, even though pyrimidine and thiazole derivatives are present,[98] or an opened thiazole ring.[99]

Thiamine intermediates show rapid reaction with the quaternary form of the thiazole, but slow reaction with the free form. A study of synthetic thiazoles showed a purple-red like thiamine only with its inactive isomer, 3-(6′-amino-4′-ethyl pyrimidyl-5′)-4-methyl-5-β-hydroxyethylthiazolium chloride hydrochoride.

In the absence of interfering substances the reaction is 75 per cent completed in 30 minutes and complete in 13 hours. Undue alkalinity destroys the vitamin. Separation of the thiamine is often necessary due to many materials inhibiting the reaction. Zeolite removes thiamine by sorption [100] rather than by base exchange.[101] Superfiltrol [102] and acid clay [103] are also used. Salts, nonaqueous solvents, and large concentrations of other organic material interfere with quantitative sorption. Interference by ascorbic acid can be prevented.[104] Extracts from leaves contain an interfering substance.[105] Even small amounts of iron may interfere slightly.[106]

Organic hydroxy compounds such as phenol, methanol, ethanol, and benzyl alcohol increase the sensitivity of the reaction but large amounts of phenol prevent the reaction unless larger quantities of reagent are used. Benzyl alcohol and phenol are excellent extractants,[107] but the latter contains the product as a difficultly-recoverable phenolate.[108]

The product is obtained from free thiamine but not from the phosphorylated form. Thiamine is present in biological samples as the free

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[98] Robert R. Williams, Robert E. Waterman, John C. Kereztesy, and Edwin R. Buchanan, *J. Am. Chem. Soc.* **57**, 536-7 (1935).

[99] Hans T. Clarke and S. Gurin, *Ibid.* **57**, 1876-81 (1935).

[100] David Melnick and Henry Field, Jr., *J. Biol. Chem.* **123**, lxxxiii (1938).

[101] L. R. Cerecedo and D. J. Hennessy, *J. Am. Chem. Soc.* **59**, 1617-9 (1937); J. C. Whitehorn, *J. Biol. Chem.* **56**, 751-64 (1923).

[102] A. D. Emmett, Gail Peacock, and Raymond A. Brown, *Ibid.* **135**, 131-8 (1940).

[103] Yosita Sakurai, T. Inagaki, and S. Omori, *J. Agr. Chem. Soc. Japan* **16**, 331-9, *Bull. Agr. Chem. Soc. Japan* **16**, 79 (1940); Yosita Sakurai, *Bull. Inst. Phys. Chem. Research (Tokyo)* **20**, 281-4 (1941).

[104] M. E. Auerbach, *J. Am. Pharm. Assn.* **29**, 313-16 (1940); Mario Lodi, *Chimica e industria* (Milan) **27**, 80-2 (1945).

[105] Gunnar Ågren, *Acta Physiol. Scand.* **10**, 381-8 (1945).

[106] R. R. Sealock and R. L. Goodland, *J. Biol. Chem.* **154**, 63-8 (1944).

[107] R. D. Greene and A. Black, *J. Am. Chem. Soc.* **59**, 1395-9 (1937).

[108] Daniel Melnick, Henry Field, Jr., and William D. Robinson, *J. Nutrition* **18**, 593-610 (1939).

amine and the phosphoric esters,[109] and the phosphoric-acid esters are as biologically active as the vitamin. The pyrophosphoric ester is cocarboxylase, the coenzyme of carboxylase. In yeast as much as 75 per cent may be esterified. Cocarboxylase hydrolyzes in 1:10 hydrochloric acid to thiamine monophosphate which is resistant to acid hydrolysis. Alkaline hydrolysis is impractical because of the thiamine destruction. Kidney phosphatase[110] hydrolyzes to the free vitamin. Incubation with takaphosphatase[111] at 45° in the presence of the enzyme from yeast liberates the thiamine within 12 hours. Takadiastase is effective at 45-50° in 2 hours.[112]

There are other diazo reagents. Sulfanilic acid is used. The rather general reaction is improved in specificity by the presence of formaldehyde.[113] Among the other amines, diazotized naphthylamines and aniline produce red with thiamine; histidine gives yellow to orange. *p*-Aminoacetanilide gives colors much like those from *p*-aminoacetophenone, but the reagent hydrolyzes in acid solution in the dark to give a red color. Other diazotized amines used are 2,4-dichloroaniline,[114] ethyl-*p*-trichloroacetaminobenzoate,[115] *p*-nitroaniline, and 2-(*p*-aminophenylsulfamido)pyridine.[116] Other reagents are *p*-dimethylaminobenzaldehyde[117] and 2,6-dibromoquinonechloroimide.[118]

Aminonaphthosulfonic acid, sulfuric acid, and ammonium molybdate give a blue color when reacted with thiamine.[119] If phosphate is present, ash another sample, analyze similarly, and apply a suitable correction.

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109 K. Lohmann and P. Schuster, *Biochem. Z.* **294**, 188-214 (1937); Severo Ochoa and Rudolf A. Peters, *Biochem. J.* **32**, 1501-15 (1938).

110 Henry Tauber, *J. Biol. Chem.* **123**, 499-506 (1938).

111 H. W. Kinnersley and R. A. Peters, *Biochem. J.* 32, 1516-20 (1938).

112 Nirmalendu Nandi, Haripada Chattopadhyay and Sachchidananda Banerjee, *Indian J. Physiol.* **3**, 50-5 (1949).

113 V. A. Deviatnin, *Compt. rend. acad. sci. URSS* (NS) **4**, 67-71 (1936); Paul Meunier and Claude Blancpain, *Compt. rend. soc. biol.* **208**, 768-70 (1939); *Bull. soc. chim. biol.* **21**, 649-64 (1939).

114 Harry Willstaedt and Franz Bárány, *Enzymologia* **2**, 316-20 (1938).

115 Ernest R. Kirch and Olaf Bergeim, *J. Biol. Chem.* **143**, 575-88 (1942); *J. Am. Pharm. Assoc.* **32**, 56-8 (1943).

116 A. D. Marenzi, *Anales farm. bioquím.* (Buenos Aires) **11**, 115-18 (1940); A. D. Marenzi and F. Vilallonga. *Ibid.* **13**, 24-9 (1942); C. S. Runti, *Intern. Z. Vitaminforsch.* **19**, 282-302 (1948).

117 Henry Tauber, *Science* **86**, 594 (1937); I. Panshina-Trufanova, *Biokhimaga* **1**, 597-602 (1936).

118 Harry W. Raybin, *Science* **88**, 35 (1938).

119 Gilberto Guimaraes Villela and Aluisio M. Leal, *Ibid.* **90**, 179 (1939).

Thiamine is estimated by growth of *Phycomyces blakesleeanus*[120] and subsequent determination of the pyruvic acid formed by its reaction product with 2,4-dinitrophenylhydrazine (Vol. III, page 356).

For relatively concentrated solutions of thiamine determination as the reineckate is preferred.[121] Interference by niacin, niacinamide, and pyridoxine is avoided by buffering to pH 4.5. Interference by some cyclic amines and by quaternary ammonium compounds other than thiamine is unavoidable and their presence precludes use of the method. Decomposition products of thiamine do not react.

Oxidation of thiamine produces a yellow pigment, thiochrome, which shows an intense fluorescence in ultraviolet light, such as by 365 mμ. The usual oxidizing agent is potassium ferricyanide. The thiochrome is extracted into isobutanol for reading.[122] Thiamine is also estimated in urine as thiochrome without extraction with butanol.[123] Oxidation is with potassium ferricyanide and alcoholic hydrogen peroxide. To avoid interference by other fluorescing substances the thiamine is sometimes sorbed on zeolite and eluted with ammonium nitrate solution.[124] As with the derivative of *p*-aminoacetophenone the phosphate esters of

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[120] R. Utiger, *Bull. soc. chim. biol.* **31**, 238-49 (1949).

[121] F. J. Bandelin and J. V. Tuschoff, *Anal. Chem.* **25**, 1198-1200 (1953).

[122] R. A. Peters, *Nature* **135**, 107 (1935); George Barger, F. Bergel, and A. R. Todd, *Nature* **136**, 259 (1935); *Ber.* **68**, 803 (1937); J. Goudsmit and H. G. K. Westenbrink, *Nature* **139**, 1108-9 (1937); Magnus A. Pyke, *Biochem. J.* **31**, 1958-63 (1937); Walter Karrer and Ulrich Kubli, *Helv. Chim. Acta* **20**, 369-73 (1937); F. Widenbauer, O. Huhn, and G. Becker, *Z. ges. exptl. Med.* **101**, 178-86 (1937); Hans Otto and F. Rühmekorb, *Klin. Wochschr.* **17**, 1246-7 (1938); K. Ritsert, *Deut. med. Woschschr.* **64**, 481-4 (1938); George M. Hills, *Biochem. J.* **33**, 1966-79 (1939); R. G. Booth, *J. Soc. Chem. Ind.* **59**, 181-4 (1940); L. C. Norris and J. C. Bauernfeind, *Food Research* **5**, 521-32 (1940); Leslie J. Harris and Y. L. Wang, *Biochem. J.* **35**, 1050-67 (1941); R. T. Conner and G. J. Straub, *Ind. Eng. Chem., Anal Ed.* **13**, 385-8 (1941); Akiji Fujita, Tetu Asari, and Keizaburo Dohi, *J. Biochem.* (Japan) **33**, 339-57 (1941); Akiji Fujita and Danji Matsukawa, *Ibid.* **33**, 385-408 (1941); O. D. Bird, J. M. Vandenbelt and A. D. Emmett, *J. Biol. Chem.* **142**, 317-22 (1942); Melvin Hochberg, Daniel Melnick, and Bernard L. Oser, *Ibid.* **155**, 119-36 (1944); D. F. Clausen and R. E. Brown, *Ind. Eng. Chem., Anal. Ed.* **16**, 572-4 (1944); H. Williams and F. Wokes, *Quart J. Pharm. Pharmacol.* **20**, 240-57 (1947); F. G. Stock, *Analyst* **75**, 499 (1950).

[123] Otto A. Bessey, O. H. Lowry, and E. B. Davis, *J. Biol. Chem.* **195**, 453-8 (1952).

[124] Leopold R. Cerecedo and D. J. Hennessy, *J. Am. Chem. Soc.* **59**, 1617-18 (1937); Leopold R. Cerecedo and F. J. Kaszuba, *Ibid.* **59**, 1619-20 (1937); Leopold R. Cerecedo and John J. Thornton, *Ibid.* **59**, 1621-2 (1937); D. J. Hennessy and Leopold R. Cerecedo, *Ibid.* **61**, 179-83 (1939).

thiochrome are not extracted.[125] The fluorescence conforms to Beer's law up to about 0.02 mg. Uric acid can be precipitated by zinc acetate followed by sodium carbonate. Benzenesulfonylchloride inhibits the fluorescence of some interfering substances.[126] Others are best taken care of by a blank in which thiamine has been destroyed by heating with sodium carbonate.[127]

The thiochrome method is the preferred one for material of low potency as it is considerably more sensitive.[128] In visual color matching accuracy ranges from 90-106 per cent and largely depends on the preparation of the sample. Interfering substances are extracted from urine preliminary to other treatment.[129] Both ferrous and ferric iron reduce fluorescence,[130] but not in the amounts usually present in enriched cereals. However, *ferrum reductum* with acid extraction does, unless there is cystine present. The latter is reduced to cysteine and does not interfere.[131] Fine grinding permits substantial reduction of the sample: solvent ratio.[132]

Filter paper may furnish fluorescent material.[133] The amount of solvent can be reduced to increase the fluorescence.[134] Close empirical standardization of the technic is essential.[135]

Other methods include direct spectrophotometric reading[136] and reading as Prussian blue after oxidation with ferricyanide.[137] Thiamine

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125 Henry W. Kinnersley and Rudolf A. Peters, *Biochem. J.* **32**, 697-8 (1938).

126 Léon Petit, *Ann. inst. natl. rechesche agron.*, Sér. A, *Ann. agron* **1**, 41-107 (1950).

127 Yoshitsugu Nose and Toshio Toshiro, *J. Japan Biochem. Soc.* **21**, 130-4 (1949); *Ibid.* **22**, 21-8 (1950).

128 Douglas J. Hennessy, *Ind. Eng. Chem., Anal. Ed.* **13**, 216-18 (1941); R. A. Brown, Eva Hartzler, Gail Peacock, and A. D. Emmett, *Ibid* **15**, 494-5 (1943); Frank Urbon and Melvin L. Goldman, *J. Biol Chem.* **152**, 329-37 (1944); E. C. Grob, *Z. Vitaminforsch.* **17**, 98-130 (1946); cf. David Glick, *Cereal Chem.* **21**, 119-26 (1944).

129 Enrique Egana and Arnold P. Meikeljohn, *J. Biol. Chem.* **141**, 859-70 (1941).

130 P. Ellinger and M. Holden, *Biochem. J.* **38**, 147-50 (1944).

131 Saul H. Rubin, Elmer DeRitter, R. L. Schuman, and J. C. Bauernfeind, *Ind. Eng. Chem., Anal. Ed.* **17**, 136-40 (1945).

132 Winifred F. Hinman, Evelyn G. Halliday, and Margaret H. Brookes, *Ibid.* **16**, 116-20 (1944).

133 Valentine Fürst, Jr., *Tids. Kjimi, Bergvesen Met.* **2**, 39 (1942).

134 A. Mukherji, *J. Indian Chem. Soc.* **16**, 273-80 (1939).

135 Donald F. Clausen, *Anachem. News* **8**, 79-88 (1948).

136 K. H. Coward and J. O. Irwin, *Quart. J. Pharm. Pharmacol.* **14**, 329-36 (1941).

137 Aladár Jendrassik, *J. Biol Chem.* **57**, 129-38 (1923); Henry Tauber, *Mikrochim. Acta* **3**, 108-9 (1938); Fritz Reindel and Heinz Habersbrudder, *Vitamine u. Hormone* **4**, 306-14 (1943).

produces a colored reaction product with cyanogen bromide or under other conditions a fluorescent compound.[138] Pyridoxyl interferes.

Samples—*Rice polishings, wheat germ, yeast.* Take a ground sample sufficient to yield about 0.15 mg. of thiamine. Add about 25 times the weight of water at 70°, adjust the pH to 4.5 with 1:9 sulfuric acid, and maintain at that temperature under nitrogen for 30 minutes of mechanical stirring. Centrifuge and wash the residue. Transfer the thiamine solution to the apparatus shown in Figure 4.

Wash approximately 50-mesh zeolite twice with 10-volume portions of 3 per cent acetic acid for 10 minutes each. Treat with 5 volumes of 25 per cent potassium chloride solution for 15 minutes. Wash twice more with the acetic acid as before. Next wash the alkali-free zeolite by successively suspending it five times in distilled water, thrice in ethanol, once in acetone, and twice in anhydrous ether. Filter, dry at 50°, and store. Put 3 grams in the tube to give about a 100-mm. column. Put through 30 ml. of 25 per cent potassium chloride solution at pH 2 heated with the steam jacket. Then wash with 500 ml. of hot water. Release the suction and fill the system with nitrogen. Connect the desired sample to the column by the siphon for filtration and allow it to trickle through with the system under atmospheric pressure by opening stopcocks A and B. Filtration is at about 10 ml. per minute. When the siphon takes up the last of the sample add 30 ml. of water adjusted to pH 4.5 with sulfuric acid.

This should follow through separated by a bubble of gas. When the last of the sample solution is in the filter column, retain the wash solution in the siphon by opening the nitrogen reservoir and apply suction to hasten filtration. Open to the atmosphere and, with steam in the jacket, transfer the wash solution to the filter column. Follow the wash water through the siphon with 10 ml. of 2.5 per cent potassium chloride solution in 1:150 hydrochloric acid but retain in the siphon by the technic described. Pass the hot wash solution which not only removes the last of the sample but heats the zeolite to the temperature for elution.

Suspend the tube F from the supports G and siphon the solution for elution very slowly through the steam jacket with suction only at the end. The zeolite should be at about 65°. After removal of the 10-ml. of eluate, wash the zeolite with 500 ml. of hot water so that it

[138] Arthur E. Teeri, *J. Biol. Chem.* **196**, 547-50 (1952).

will be ready for reuse. Use an aliquot of the eluate for development with diazotized *p*-aminoacetophenone, diluting with potassium chloride adjusted to pH 2 if necessary.

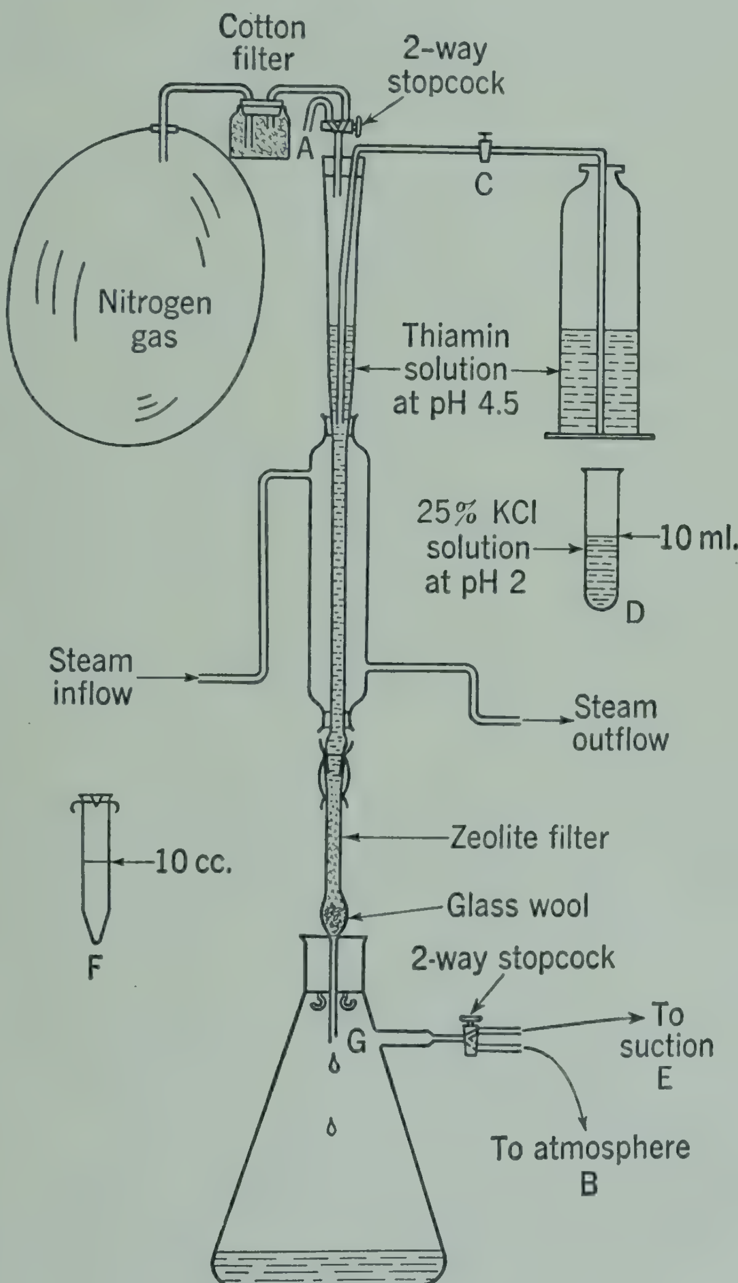

FIG. 4. Apparatus used for both quantitative absorption of thiamine by zeolite and subsequent quantitative elution

A simplified extraction apparatus shown in Figure 5 may be used with somewhat lower accuracy.

Rice. Boil 15 grams of finely ground sample for 15 minutes with 150 ml. of 1:180 sulfuric acid and a disintegrated filter paper. Stir until thin enough to boil freely. Cool and add 90 ml. of 3.16 per cent solution of barium hydroxide octahydrate. Add 1 ml. of bromocresol

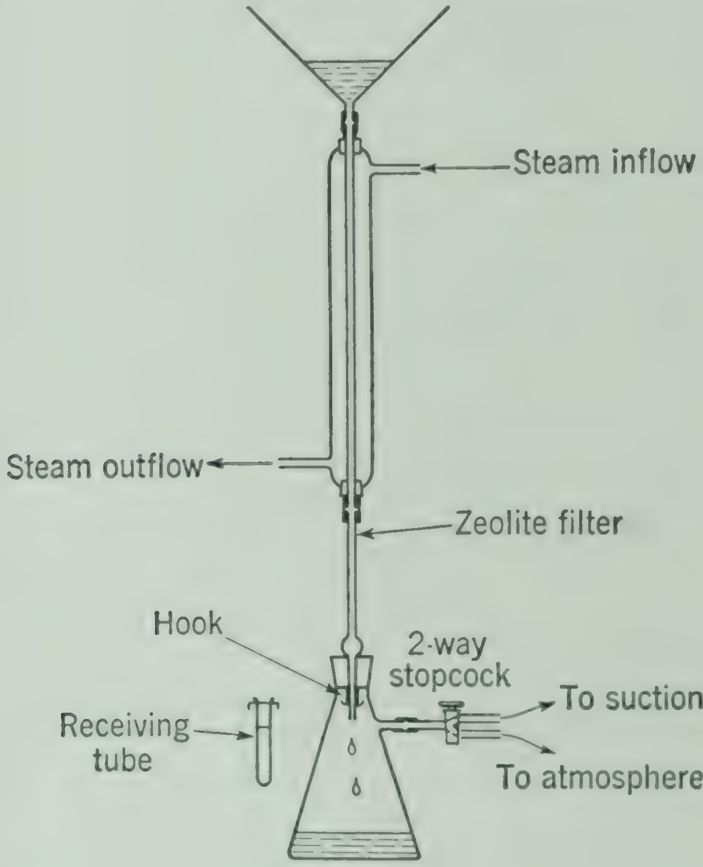

Fig. 5. Simplified apparatus for sorption and elution of thiamine

green indicator solution and follow with 0.8 per cent sodium hydroxide solution until a definite blue color is obtained. This is pH 5.0-5.4. Dilute to 240 ml., mix, and filter on a fluted filter. The indicator is largely sorbed on the precipitate. Determine by diazotized *p*-amino-acetophenone reagent on a portion of the filtrate at once, using the optional technic.

Cereal products.[139] Weigh a 20-gram sample and add 150 ml. of 1:700 sulfuric acid. Reflux for 30 minutes, cool, and add 10 ml. of 2.5 per cent sodium acetate trihydrate solution to adjust the pH to 4.5. Add 1 gram of takadiastase or Mylase-P and incubate the suspension overnight at 38° or for 3 hours at 45-50°. Cool to 20°and dilute with water to 200 ml. Centrifuge or filter to remove insoluble material. Continue as for rice polishings, starting at "Transfer the thiamine solution to . . . ."

Flour.[140] Stir a 25-gram sample in 100 ml. of 1:20 hydrochloric acid and leave overnight. Filter the supernatant liquid and to a 40-ml. aliquot add 50 mg. of Superfiltrol. Shake mechanically for 30 minutes and centrifuge. Wash the sorbate with 5 ml. of 1:20 hydrochloric acid solution and centrifuge. Again shake the supernatant liquid with 50 mg. of Superfiltrol. Transfer to the same tube, centrifuge, and discard the supernatant fluid. Wash the sorbate with 1:20 hydrochloric acid and discard the washings. Add 1 ml. of water to the sorbate and then add 2 drops of bromocresol green solution. Adjust the pH to 5 by the addition of 2 per cent sodium hydroxide solution. Transfer the sorbate, wash the tube with 60 per cent ethanol, and add the washings to the sorbate. Dilute to a known volume and develop the color in an aliquot with diazotized *p*-aminoacetophenone.

Flour premixes.[141] Such finely divided samples do not require hot extraction or enzyme treatment. Mix 10 ml. of 1:360 sulfuric acid with 25 ml. of 10 per cent sodium acetate trihydrate solution, and for use dilute 1:5 with water. Mix a sample with such a volume of this diluted buffer that the final solution will contain 0.0001-0.0002 mg. of thiamine per ml. and shake mechanically for 30 minutes. Check roughly that the buffer with sample falls in the pH range 5.5-6.5. Develop an aliquot as thiochrome or with diazotized *p*-aminoacetophenone. This sample is also suitable for estimation of riboflavin.

Vegetables.[142] Macerate 25 grams in a blender with 150 ml. of 1:360 sulfuric acid. Heat in boiling water for a half hour with frequent agitation during the first 5 minutes and thereafter every 5 minutes

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139 Melvin Hochberg, Daniel Melnick, and Bernard L. Oser, *Cereal Chem.* **22**, 83-90 (1945); cf. L. P. van der Mijil Dekker, *Chem. Weekblad* **43**, 532-3 (1947).

140 B. S. Platt and G. E. Glock, *Biochem. J.* **37**, 439-43 (1943).

141 Elmer de Ritter and Saul H. Rubin, *Anal. Chem.* **19**, 243-8 (1947); Saul H. Rubin *et al., J. Cereal Chem.* **25**, 52-60 (1948).

142 James C. Moyer and Donald K. Tressler, *Ind. Eng. Chem., Anal. Ed.* **14**, 788-90 (1942).

for 0.5 hour. Cool below 50° and add 25 ml. of 20 per cent sodium acetate solution containing 0.5 gram of freshly added Clarase. Incubate at 50° for 2 hours to hydrolyze thiamine phosphates. Dilute to 500 ml. Filter, discarding the first 10 ml., and develop by the thiochrome method.

Powdered liver extract. Dissolve in the minimal volume of water, saturate with sodium sulfate, and concentrate *in vacuo* to a syrup. Add benzyl alcohol and continue to heat until the last moisture has been driven off. Separate the benzyl-alcohol extract and wash the residue with the same solvent. Add 5 volumes of ether to the benzyl alcohol solution and extract the thiamine with water adjusted to about pH 3.5 with sulfuric acid.

Continue as for rice polishings, etc., starting at "Transfer the thiamine solution to . . . ."

Pharmaceutical ampoules.[143] Dilute with water so that each ml. contains about 0.1 mg. of thiamine and develop with diazotized *p*-aminoacetophenone. For development as the reineckate dilute to about 0.25 mg. per ml.

Elixirs. Dilute as for the contents of ampoules. If the full color does not develop, elute the sample as for rice polishings, starting at "Transfer the thiamine solution to . . . ."

Tablets. Pulverize 10 tablets. Weigh out a sample containing about 1 mg. of thiamine, transfer to 10-ml. of 50 per cent ethanol, and let stand at 60° for 10 minutes with occasional mixing. Cool, mix well, and centrifuge until clear. Use an aliquot with diazotized *p*-aminoacetophenone.

Alternatively,[144] boil a sample containing 10 mg. of thiamine with 5 ml. of 1:10 hydrochloric acid for 4 minutes, cool, neutralize with 4 per cent sodium hydroxide solution, and dilute to 100 ml. for development with the same reagent.

For development as the reineckate digest an aliquot of the powdered tablets containing 5-10 mg. of thiamine with 20 ml. of the acetate buffer for pH 4.5 described under procedure (page 96). Thirty minutes around 100° is usually satisfactory. Filter and wash the residue with water. Dilute to 25 ml. and use 10 ml. for development. Pick up the procedure at "Add 5 ml. of 2 per cent ammonium reineckate. . . ."

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143 M. E. Auerbach, *J. Amer. Pharm. Assn.* **29**, 313-16 (1940).

144 C. W. Ballard and E. J. Ballard, *J. Pharm. Pharmacol.* **1**, 330-3 (1949).

Hard-filled capsules. Remove the powder and treat as powdered tablets from "For development as the reineckate . . . ."

Soft gelatin capsules. Cut open and macerate in a mortar with several 15 ml. portions of ether until the oil is completely dissolved. Extract the combined ether extracts with four successive 5-ml. portions of acetate buffer for pH 4.5 (page 96). Filter the combined acid extracts, wash the filter with water, and dilute to 25 ml. Use a 10-ml. aliquot and pick up the procedure at "Add 5 ml. of 2 per cent ammonium reineckate . . . ."

Milk.[145] Dilute a 20-ml. sample with 10 ml. of water and add 1 ml. of 1:3 hydrochloric acid. Heat at 90° for 5 minutes and add 12 grams of anhydrous sodium sulfate. Stir for 3 minutes at that temperature to coagulate. Filter through a retentive paper and extract with 20, 10, and 10 ml. of isobutanol to remove interfering fluorescing substances. Use an aliquot of the clear liquid for development as thiochrome.

Tissue extracts and blood. Precipitate the proteins by adding sufficient trichloroacetic acid to make it 2 per cent. Centrifuge and develop an aliquot of the upper layer.

Urine.[146] Collect a 24-hour sample in a bottle containing 10 ml. of toluene as preservative and 20 ml. of 1:9 sulfuric acid to keep the reaction at a pH of less than 3. Under these conditions of vitamin stability the sample may be stored for a month. Adjust the pH to 5, using Congo red and bromocresol-green paper as indicators and transfer to the apparatus shown in Figure 6. Add 20 grams of anhydrous sodium sulfate to prevent the residue from becoming gummy and difficult to extract. Add a few small glass beads to prevent bumping. Concentrate on a steam bath. If there is foaming, add a few drops of benzyl alcohol. As soon as salts begin to precipitate, add 80 ml. of benzyl alcohol and continue to concentrate until the aqueous phase has almost completely disappeared. Leave a few ml. of water to avoid overheating.

Transfer the mixture to a 250-ml. bottle, centrifuge, and decant the benzyl alcohol solution, avoiding decanting any of the aqueous phase. Agitate the residue vigorously with 40 ml. of benzyl alcohol and separate these washings.

Shake the combined extract and washings with 125 ml. of 1:100 sulfuric acid and 500 ml. of ether. Draw off the aqueous layer into a

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[145] M. Slezic and Th. Asher, *Arch. wiss. prakt. Tierheilk* 77, 170-1 (1941).

[146] Cf. Akiji Fujita and Danji Matsukawa, *J. Biochem.* (*Japan*) 35, 79-87 (1950).

separatory funnel. Extract the solvent layer again with 125 ml. of 1:100 sulfuric acid and add this to the aqueous phase. Send the solvent layer to recovery and extract the aqueous phase with 500 ml. of ether which may be reused for the same purpose. Evaporate ether from the

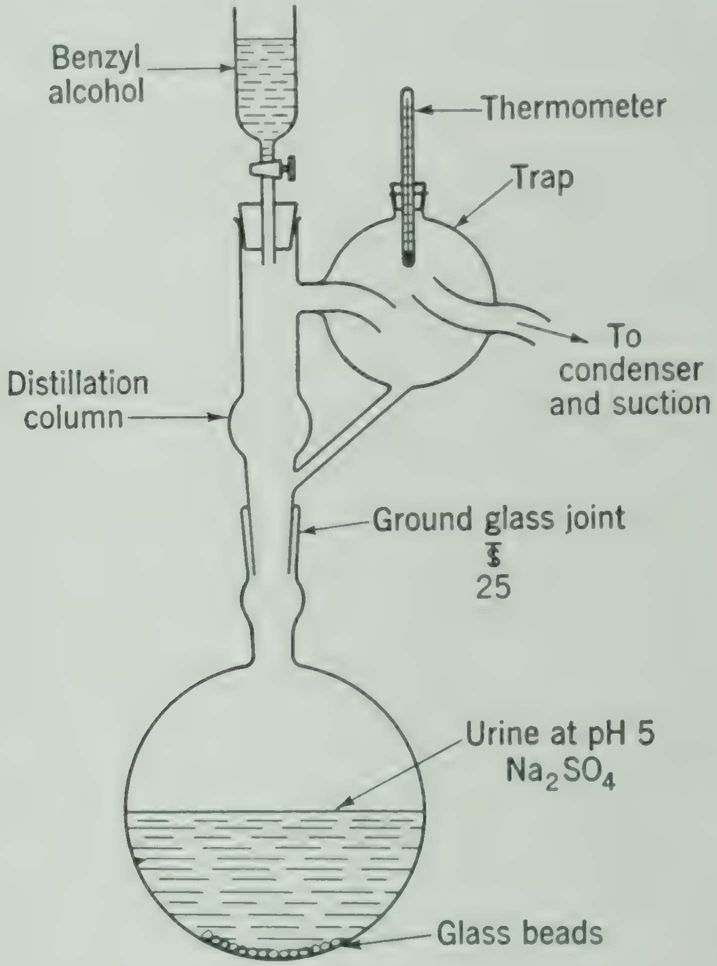

FIG. 6. Distillation unit for the concentration of urine *in vacuo*

aqueous phase by distillation *in vacuo*. Continue as for rice polishings, etc., starting at "Transfer the thiamine solution to . . . ."

Alternatively, adjust to pH 4.5 for sorption and proceed as for rice polishings, etc.,[147] starting at "Transfer the thiamine solution . . . ." Develop the final eluted sample as thiochrome.

[147] Robert E. Johnson, Frederick Sargent, Paul F. Robinson, and Frank C. Consolazio, *Ind. Eng. Chem., Anal. Ed.* 17, 384-6 (1945).

As a micro method, add 0.1 gram of oxalic acid per 100 ml. to freshly voided urine and store in amber bottles. Preliminary treatment is necessary only if the patient is receiving atabrine or quinine. In that case extract with isobutanol, treat with a very small amount of activated carbon, and filter for use as sample.

To a 2-ml. sample in a glass-stoppered test tube add about 0.2 gram of acid-washed zeolite, previously separated from fine particles by sedimentation. Shake a few times. At this stage the sample may stand if desired. Add approximately 8 ml. of 1:100 acetic acid and shake briefly. A slight turbidity has no significance. Discard the liquid and wash the zeolite with 8 ml. of 1:100 acetic acid. If washing is not complete, a silvery blue fluorescence will be superimposed on that of thiochrome. There is no loss of thiamine in washing.[148] Add 0.5 ml. of 25 per cent potassium chloride solution to extract the thiamine. After 30 seconds use as sample for development as thiochrome without removal of the zeolite.

Solids. Reflux a finely divided sample for 20 minutes with 5-20 parts of 2 per cent acetic-acid solution. Cool, centrifuge, and re-extract. If gelation prevents centrifuging, add sufficient ethanol to make it 30 per cent of the sample. For base-exchange or enzymatic hydrolysis this must be distilled *in vacuo* before further use of the combined extracts. Dilute to a known volume for direct use of an aliquot or separation of thiamine by sorption, or release by enzyme action. In any case develop by oxidation to thiochrome.

Separation by sorption. Use a tube of 7 mm. inside diameter with an outer steam jacket of about 40 cm. The inner jacket is constricted at the lower end and holds a plug of glass wool. Above this is placed a 20-cm. column of alkali-free 30-mesh zeolite. At the upper end of the tube is a funnel and at the lower end is a two-way stop-cock for gravity flow and suction.

If the zeolite is new, treat by alternately passing through four 15-ml. portions of boiling 2 per cent acetic-acid solution and 25 per cent potassium chloride solution. While doing this, pass steam through the outer jacket. Next pass three 15-ml. portions of boiling water through the column. The tube is then ready for use.

Adjust the pH of 5-20 ml. of sample solution containing up to 0.01 mg. of thiamine to 4-4.5 with acetic acid. Bring to a boil and introduce

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148 When this preparation of sample is for determination of niacinamide the washings must be rigidly standardized as there is loss in washing.

into the zeolite which is kept near 100°. Pass the solution through the bed in 3-5 minutes. Follow by three 5-ml. portions of boiling water and then apply suction to draw excess water from the tube.

To elute the vitamin from the zeolite, run 25 per cent aqueous potassium chloride through the column at the rate of 1 ml. per minute. Collect 10-25 ml. of effluent. Assay 5 ml. aliquots of this by development as thiochrome, using 0.05 ml. of potassium ferricyanide solution.

Enzyme action. To prepare the enzyme, shake or stir a ground, defatted, beef-kidney sample with an equal weight of 1 per cent sodium chloride solution for 2 hours. Centrifuge and place the centrifugate in the refrigerator. Re-extract the residue in the same way. Stir the combined extracts with enough 300-mesh bentonite to form a thin paste and centrifuge. Filter the centrifugate to obtain about 60 per cent of the original extract as a clear liquid. Stir with one-half volume of acetone and centrifuge off the precipitate. Add a like portion of acetone to the liquid, stir, and again collect the precipitate as before. Dry the combined precipitates in a vacuum desiccator over sulfuric acid. Powder the dry residue and preserve in a brown, stoppered bottle.

Adjust the solution containing up to 0.01 mg. of thiamine to pH 6.5-7. Add 20-80 mg. of the powdered enzyme and maintain at 37° for 3 hours. Acidify the hydrolyzate with a drop of glacial acetic acid. Boil, cool, and centrifuge. If the purity permits, use all or an aliquot as sample for oxidation to thiochrome. If necessary separate the thiamine by sorption and elution before development.

Elimination of interference by ascorbic acid.[149] To each volume of sample add an equal volume of ethanol followed by bromine water, drop by drop, until a definite yellow is obtained. Add a drop of 4 per cent sodium salicylate solution to destroy excess bromine and then 2 drops of saturated calcium chloride solution. Develop with diazotized *p*-aminoacetophenone and allow for the reagents added in destruction of ascorbic acid. Alternatively titrate the ascorbic acid with iodine solution [150] or precipitate it with lead acetate.[151]

Samples containing reduced iron. Prepare a solution of cystine in 1:6 sulfuric acid and dilute to 40 volumes with water. The cystine content should be such that a minimum of 13 mg. are present per mg. of reduced iron. Add to the sample to give 0.0001-0.002 mg. per ml.

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[149] M. E. Auerbach, *J. Am. Pharm. Assoc.* 29, 313-16 (1940).

[150] Mario Lodi, *Chimica e industria* (Milan) 27, 80-2 (1945).

[151] Benjamin Alexander and J. Elliot Levy, *J. Biol. Chem.* 146, 399-406 (1942).

and heat for 45 minutes at 100° with occasional shaking. Develop an aliquot as thiochrome or sorb on zeolite, elute, and develop with diazotized *p*-aminoacetophenone. This sample is also suitable for estimation of riboflavin.

Procedure—*By diazotized p-aminoacetophenone.* First prepare the reagent as follows. *Solution A:* Dissolve 3.18 grams of *p*-aminoacetophenone in 45 ml. of concentrated hydrochloric acid and dilute to 500 ml. with water. Store in a glass-stoppered flask out of strong light. It is stable for about 6 months. *Solution B:* A 4.5 per cent aqueous solution of sodium nitrite is stable for about a month under refrigeration. *Solution C:* Dissolve 20 grams of sodium hydroxide in 600 ml. of water. Add 28.8 grams of sodium bicarbonate and dilute to 1 liter.

Mix one part each of Solutions A and B at 0° with mechanical agitation. After 10 minutes add 4 more parts of Solution B to the mixture. Stir at 0-5° for at least 20 minutes. This diazotized solution is stable for about 12 hours at 0°. Add 20 ml. of this solution to 275 ml. of Solution C with mechanical stirring. A purple color forms which disappears after 5-10 minutes.

Adjust the pH of the sample solution to 5-6 and pipet 3 ml. containing 0.03-0.3 mg. of thiamin per ml. into 3 ml. of a 0.5 per cent solution of phenol in ethanol. Add 1 drop of thymol blue indicator solution. Bubble a fine stream of nitrogen gas through the solution and at the same time add 4 per cent sodium hydroxide solution dropwise until a faint but positive blue color appears. Add 6 ml. of the prepared reagent. Stopper and allow to stand overnight at room temperature.

Add 2 ml. of xylene and shake vigorously for 90 seconds. Residual color in the sample is not due to thiamine . If red color adheres to the precipitated zeolite, often present in biological samples, add 5 ml. of 1:4 sulfuric acid to the aqueous layer, stopper, and shake for 20 seconds. Add 10 ml. of 15 per cent sodium hydroxide solution and again shake for 20 seconds. Centrifuge the xylene layer at 520-540 mμ against a reagent blank.[152] The xylene may then be decolorized by activated carbon for reuse.[153]

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[152] Benjamin Alexander and J. Elliot Levy, *Ibid.* **146**, 399-406 (1942); Gilberto Guimeraes Villela, *Rev. brasil, biol.* **2**, 67-71 (1942); Henrique Tastaldi, *O Hospital* **23**, 537-52 (1943); Mario Lodi, *Chimica e industria* (Milan) **27**, 80-2 (1945); Melvin Hochberg, Daniel Melnick, and Bernard L. Oser, *Cereal Chem.* **22**, 83-90 (1945).

[153] Morton Pader, *Ind. Eng. Chem., Anal. Ed.* **15**, 25 (1943).

Optional technic. To 160 ml. of extract add 100 ml. of 0.04 per cent phenol in ethanol. Add 4 drops of 0.05 per cent phenol red solution in 10 per cent ethanol. Add 0.8 per cent sodium hydroxide solution until the color changes from yellow to red. Separately mix 1.6 ml. of 23 per cent sodium nitrite solution, 1.6 ml. of 2.5 per cent *p*-aminoacetophenone solution in 9:91 hydrochloric acid, 20 ml. of water, and 50 ml. of a solution containing 6 per cent of sodium hydroxide and 8.4 per cent of sodium bicarbonate. Add this mixture and follow with 8 ml. of 1:1 xylene-isooctane. Shake every 15 minutes for an hour and separate a portion of the upper layer. Dry with anhydrous sodium sulfate and read at 520 mμ.

As the reineckate. As buffer for pH 4.5 mix 114 ml. of acetic acid with 86 ml. of 1.64 per cent solution of anhydrous sodium acetate or 2.72 per cent solution of the trihydrate. Add a solution containing 2-5 mg. of thiamine to 10 ml. of the buffer. Add 5 ml. of 2 per cent ammonium reineckate in absolute methanol. After 30 minutes filter on fritted glass and wash with 3, 3, and 3 ml. of a 1:500 dilution of the ammonium reineckate reagent with water. Dissolve the precipitate in successive 2-ml. portions of acetone and dilute to 10 ml. Read at 525 mμ against a reagent blank.

As thiochrome. Macro. As oxidizing solution dilute 4 ml. of freshly prepared 1 per cent potassium ferricyanide solution to 100 ml. with 15 per cent sodium hydroxide solution. Prepare acidified potassium chloride solution by addition of 0.85 ml. of concentrated hydrochloric acid to 100 ml. of 25 per cent potassium chloride solution.

Dilute a portion of sample containing 0.0001-0.002 mg. of thiamine to 5 ml. with the acidified potassium chloride solution. Add 3 ml. of the oxidizing solution and mix. At the end of 2 minutes add 13 ml. of isobutanol and shake for 90 seconds. Centrifuge to separate the layers. Remove the isobutanol layer and, if cloudy, dry with about 2 grams of anhydrous sodium sulfate. At the same time prepare a blank in which the oxidizing solution has been replaced with 15 per cent sodium hydroxide solution. Compare the extracted sample with the extracted blank for the presence of bound thiamine and if present repeat, with release by enzyme action.

Read the fluorescence at 350-400 mμ and compare with a calibration curve based on oxidized thiamine solutions. The isobutanol can be recovered for reuse by treatment with activated carbon.

Micro. To a 0.5-ml. sample in 25 per cent potassium chloride solu-

tion add 0.1 ml. of fresh aqueous 0.25 per cent potassium ferricyanide solution. Mix and add 5 drops of 15 per cent aqueous sodium hydroxide solution. Thiochrome forms at once and is stable for 0.5 hour. Add 2 ml. of isobutanol and shake vigorously. Let stand or centrifuge and read in a fluorimeter.

By cyanogen bromide. Dilute the sample to contain not over 0.005 mg. in 10 ml. Add 5 ml. of a buffer solution containing 15 ml. of 15 per cent sodium hydroxide solution, 5 ml. of 85 per cent phosphoric acid, and 175 ml. of ethanol, diluted to 1 liter and adjusted to pH 6.6. Add 5 ml. of 4 per cent cyanogen bromide solution. Read fluorescently after 30 minutes at room temperature. Use Corning 597 and 306 and sodium fluorescein at 0.01 mg. per 100 ml. of 0.004 per cent sodium hydroxide solution.

Barbituric acid and barbiturates, Barbitals

Barbituric acid and many of its derivatives react with cobalt in anhydrous alkaline media to give a blue color.[154] Varied cobalt salts used include the nitrate and acetate. Alkalies are more varied and have embraced barium hydroxide, lithium hydroxide, piperidine, diethylamine, isopropylamine, etc. The color develops promptly and is stable for at least 30 minutes. Moisture causes the color to fade. Accuracy can be to better than 2 per cent.[155] It is essential that the same barbiturate as is being determined be used as standard.[156] With isobutylamine as the alkali[157] the color reaction is quantitative with 5,5-disubstituted and 5,5,N-trisubstituted forms. The color is unstable

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[154] J. J. L. Zwikker, *Pharm. Weekblad* **68**, 975-83 (1931); *Ibid.* **69**, 1178-88 (1932); J. M. Dille and T. Koppanyi, *J. Am. Pharm. Assn.* **23**, 1079 (1934); C. R. Linegar, J. M. Dille and T. Koppanyi, *Ibid.* **24**, 847 (1935); H. Oettel, *Arch. Pharm.* **274**, 1-10 (1936); John T. Brundage and Charles M. Gruber, *J. Pharmacol.* **59**, 379-92 (1937); Henri Griffon and Roger Le Breton, *J. pharm. chim.* **28**, 49-60 (1938); *Ann. pharm. franc.* **5**, 393-403 (1948); D. S. Belenits'Ka, *Ukrain. Gosudarst. Ist. Eksptl. Farm.* (Kharkov),, *Konsul'tatsionnye Materialy*, **1939**, No. 3, 72-4; Theodore Koppanyi, James M. Dille, William S. Murphy, and Stephen Krop, *J. Am. Pharm. Assn.* **23**, 1047-9 (1939); G. A. Levvy, *J. Am. Pharm. Assoc.* **34**, 73-7 (1940); M. W. Green, F. P. Vietch and T. Koppanyi, *Ibid.* **32**, 309 (1943); K. Kámen, *Chem. Listy* **38**, 119-24 (1944); J. Raventos, *Brit. J. Pharmacol.* **1**, 210-14 (1946); Leland N. Mattson and Wendell L. Holt, *J. Am. Pharm. Assn.* **38**, 55-7 (1949).

[155] R. F. Krause and Richard F. Riley, *J. Pharmacol.* **71**, 287-92 (1941).

[156] Eugene L. Cohen, *Am. J. Pharm.* **118**, 40-62 (1946).

[157] H. Baggersgaard-Rasmussen and B. Jerslev, *Dansk. Tids. Farm.* **25**, 29-49 (1951).

with 5-mono, N-mono, and 5,N-disubstitution. None develops with N,N′-di, 5,N,N′-tri, and 5,5,N,N′-tetrasubstitution.

N-Methyl-cyclohexenyl-methyl barbiturate, *Evipan*, gives the general reaction of barbiturates with cobalt in anhydrous alkaline solution, but to avoid turbidity in the final solution the sample must be acidified for chloroform extraction. The sensitivity is less than of most barbiturates.

Barbiturates extracted from chloroform into 2 per cent sodium hydroxide are read at pH 10 in the ultraviolet at 255 mμ, or 240 mμ.[158] Any background absorption can be read in the sample after acidification to pH 2, when the barbituric acid derivative gives no color.[159]

At pH 9.8-10.5 the maxima at 260 mμ for different barbiturates are the same. In more strongly alkaline solution differences in maxima are characteristic of all but the N-methyl and thio derivatives.[160] The method will determine amounts as low as 0.1 mg. per 100 ml. of blood or 0.3 mg. per 100 grams of tissue. Salicylates are not extracted at the pH used. Dicoumarol, dilantin, and sulfadiazine are extracted but do not interfere in reading in alkaline solution in the ultraviolet because they do not give the characteristic difference in absorption at the two pH levels.

Samples—*Pharmaceutical preparations.* Render a sample containing about 40 mg. of barbiturates acid with a few drops of 1:10 sulfuric acid. Extract with 10, 10, and 5 ml. of chloroform. Evaporate the combined extracts and dry the residue at 100° for two hours. Take up in 2 ml. of chloroform and develop by cobalt acetate and isopropylamine.

Tissue. Allow ground organs to stand for 24 hours with 5 per cent sodium hydroxide or potassium hydroxide solution. Shake the resulting solution with an equal volume of 10 per cent cupric sulfate solution. Filter and extract with 10 volumes of choloroform. Concentrate to an appropriate volume and develop the blue color by means of cobalt acetate and isopropylamine. The method is not applicable to the brain and spinal cord.

Blood. Shake a 10-ml. sample with 10 ml. of chloroform for 5 minutes. Remove the chloroform layer and add 3 ml. of 1:30 hydro-

158 Leo R. Goldbaum, *J. Pharmacol. Exptl. Terap.* **94**, 68-75 (1948).

159 J. T. Walker, R. S. Fisher, and J. J. McHugh, *Am. J. Clin. Path.* **18**, 451-6 (1948); Metry Bacila and José A. Alcaide, *Rev. col. farm. nacl.* (Rosario, Arg.) **15**, 6-9 (1948); G. V. R. Born, *Biochem. J.* **44**, 501-5 (1949); T. C. Gould and C. H. Hine, *J. Lab. Clin. Med.* 34, 1462-70 (1949); Per Lous, *Acta Pharm. Toxicol.* **6**, 227-34 (1950).

160 Leo R. Goldbaum, *Anal. Chem.* **24**, 1604-7 (1952).

chloric acid. Shake for 2-3 minutes and filter the chloroform layer. Evaporate on a water bath to 2 ml. Develop color by means of cobaltous nitrate and piperidine.

Alternatively deproteinize blood with phosphotungstic acid. Shake with acid-washed activated carbon to sorb the barbiturate. Extract the barbiturate with a mixture of equal parts of petroleum ether and diethyl ether. Evaporate the solvents and take up the anhydrous residue in chloroform. Dilute to a known volume to develop color in an aliquot by means of cobalt acetate and isopropylamine.

As another method of treatment of blood samples, mix 20 ml. of blood with 2 grams of monosodium orthophosphate and 40 grams of anhydrous sodium sulfate. Dry and grind. Extract with a 1:1 mixture of ether and petroleum ether in a Soxhlet for 3 hours. Remove phospholipids by shaking the extract with a 3:1 mixture of activated carbon and magnesium oxide. Filter and evaporate the solvents. Take up the anhydrous residue in chloroform, dilute to a known volume, and develop color in an aliquot by cobalt acetate and isopropylamine.

Urine. Treat by any of the methods for blood.

Procedure—*By cobalt acetate and isopropylamine.* To prepare the cobalt reagent, dissolve 0.125 gram of cobalt acetate, which has been dried at 100° for 2 hours, in 100 ml. of absolute methanol. Prepare the isoproplyamine reagent by diluting 25 ml. to 100 ml. with absolute methanol.

To a 5-ml. aliquot of sample in chloroform, add 5 ml. of 0.125 per cent anhydrous cobalt acetate in absolute methanol, and 5 ml. of 25 per cent isopropylamine in absolute methanol. Dilute to 25 ml. with chloroform, mix, and read at 560 mμ. Subtract a reagent blank.

By cobaltous nitrate and diethylamine. The sample is available in either absolute ethanol or in chloroform. To 1 ml. add 1 ml. of 0.3 per cent cobaltous nitrate in absolute ethanol. Follow with 1 ml. of 22 per cent diethylamine in absolute ethanol and read against a reagent blank.

By cobaltous nitrate and piperidine. To 2 ml. of sample in chloroform, add 0.2 ml. of 1 per cent cobaltous nitrate in absolute ethanol and 0.2 ml. of 5 per cent piperidine in the same solvent. Read the violet color against a reagent blank.

By comparison of ultraviolet absorption at differing pH levels. Blood. Extract a 1-5 ml. sample with 50 ml. of chloroform. This is selected to give about 0.025 mg. per ml. in the alkaline extract below. Filter the chloroform extract and take a 40-ml. aliquot for the next

step. Extract this with 4 ml. of 1.8 per cent sodium hydroxide solution. Check this 1.8 per cent sodium hydroxide solution to be sure that 2 volumes of it added to one volume of the borate buffer as described later gives a pH of about 10.5. Discard the chloroform and centrifuge the extract to break any emulsion.

Read a portion of the clear alkaline extract at pH above 10.6 at the following wave lengths: 305, 270, 260, 252, 250, 235, 232, and 228 mμ. At 305 mμ there should be no significant difference between the sample extract and a reagent blank carried through the procedure. There is no significant absorption by barbiturates at that wave length.

As a borate buffer dissolve 12.369 grams of boric acid and 14.911 grams of potassium chloride and dilute to 200 ml. Filter any precipitate after 24 hours. Add 2 volumes of the alkaline extract of sample and blank to 1-volume portions of this buffer. This buffers at pH 10.2 ± 0.6. Read these at the series of wave lengths as before. Correct the absorptions for the blank and multiply by 1.5 to correct for dilution by the buffer. Use the difference at 260 mμ for estimation of the amount of barbiturate present.

Urine. Extract 1-5 ml. of urine of pH below 7 with 50 ml. of chloroform. Shake the extract with 5 ml. of *M* phosphate buffer for pH 7.4 (Vol. I, page 174). Proceed as for blood from "Filter the chloroform extract . . . ."

Tissue. Homogenize a weighed sample with water in an all-glass homogenizer. Make up to a known volume and use an aliquot of 5 ml. equivalent to 1 or 2 grams of tissue for extraction with 75 ml. of chloroform. In the case of brain tissue, substitute ethylene dichloride. Filter the solvent extract and take a clear aliquot for the next step. Extract this with 4 ml. of 3.6 per cent sodium hydroxide solution. Precheck this sodium hydroxide solution to be sure that when mixed in equal volume with the borate buffer the pH approximates 10.5. This higher alkalinity is to avoid turbidity which may occur with the 1.8 per cent sodium hydroxide. Complete as for blood from "Discard the chloroform and . . ." but for the absorption at 10.2-10.6 mix equal volumes of the alkaline extract and the borate buffer.

Compare the differences in absorption at the series of wave lengths. For this divide the value at each wave length by that at 260 mμ. The maximum positive difference is at 260 mμ which in the case of a barbiturate becomes negative shortly below 250 mμ. To illustrate this, a typical set of data is shown in Table 6. A difference of 0.04 at 260 mμ

TABLE 6. OPTICAL DENSITY DIFFERENCES FROM BLOOD EXTRACT

(5 ml. of blood containing 0.1 mg. of added isoamyl, ethyl barbituric acid)

	Wave Lengths, mμ									
	305	270	260	252	249	247	240	235	232	228
O.D. of 1.8 per cent NaOH extract	0.095	0.39	0.67	0.78	0.77	0.75	0.67	0.62	0.64	0.81
O.D. at pH 10.5, corrected for dilution by buffer	0.100	0.18	0.27	0.55	0.71	0.83	1.08	1.05	0.97	0.92
Differences	−0.005	+0.21	+0.40	+0.23	+0.06	−0.08	−0.41	−0.43	−0.34	−0.11
Ratio $= \dfrac{\text{O.D. differences}}{\text{O.D. differences at 260 m}\mu}$		+0.52	1.00	+0.58	+0.15	−0.20	−1.02	−1.08	−0.85	−0.28
Ratios of standard amobarbital		+0.52	1.00	+0.56	+0.12	−0.21	−1.00	−1.08	−0.88	−0.33

TABLE 7. DIFFERENTIATION OF SEVERAL REPRESENTATIVE BARBITURATES BY THEIR DIFFERENCE RATIOS

$$\left(\frac{\text{Optical density difference at given wave length}}{\text{Optical density difference at 260 m}\mu}\right)$$

	Wave Lengths, mμ								
	270	260	252	249	247	240	235	232	228
	Ratios								
Amobarbital (isoamyl, ethyl)[a]	+0.52	1.00	+0.56	+0.12	−0.21	−1.00	−1.09	−0.88	−0.33
Pentobarbital (1-methylbutyl, ethyl)[a]	+0.61	1.00	+0.40	−0.05	−0.38	−1.02	−1.00	−0.80	−0.15
Secobarbital (1-methylbutyl, allyl)[a]	+0.69	1.00	+0.30	−0.13	−0.39	−1.04	−0.89	−0.63	+0.21
Butallylonal (*sec*-butyl, β-bromo-allyl)[a]	+0.81	1.00	+0.07	−0.35	−0.63	−1.03	−0.72	+0.20	+0.96
Phenobarbital (phenyl, ethyl)[a]	+0.59	1.00	+0.36	−0.09	−0.36	−0.96	−0.84	−0.48	+0.43
Cyclopal (2-cyclopenten-1-yl, allyl)[a]	+0.80	1.00	+0.11	−0.34	−0.63	−1.10	−0.94	−0.50	+0.71

[a] Substituents in 5 position of malonylurea nucleus.

is significant. Ratios for a group of typical barbiturates are shown in Table 7, and the values for 0.02 mg. of barbiturate per ml. are given in Table 8.

TABLE 8. CHARACTERISTIC DIFFERENCES OF BARBITURATES

(All are subject to standard error of mean of ±0.02)

Barbiturate	*Difference at 260 mμ Equivalent to 0.02 mg. of Barbiturate per ml. of Alkaline Extract*
Amobarbital	0.40
Barbital	0.48
Butallylonal	0.27
Pentobarbital	0.39
Phenobarbital	0.36
Seconal	0.36

6-(N-MORPHOLINO)-4,4-DIPHENYLHEPTAN-3-ONE, PHENODOXONE

The hydrochloride of phenodoxone is *heptalgin.* When complexed with bromophenol blue it carries the dye into toluene. It is extracted into alkali for estimation of the phenodoxone.[161] It is also read in water at 292.5 mμ, or in ethanol at 294.5 mμ.[162]

Sample—*Urine.* Make a sample estimated to contain 0.1–0.6 mg. of phenodoxone distinctly alkaline with 40 per cent sodium hydroxide solution. Extract with 10, 5, and 5 ml. of toluene. Shake the combined toluene extracts with 10 ml. of bromophenol blue indicator buffered at pH 4 (Vol. I, pages 176 and 204). The toluene layer becomes yellow in proportion to the phenodoxone present. Separate the toluene layer and filter to remove any suspended droplets of indicator solution. Shake with 10 ml. of 0.4 per cent sodium hydroxide solution and read this layer at 592 mμ against a sample blank.

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[161] J. E. Page and Heather King, *Analyst* 75, 71-6 (1950).

[162] W. H. C. Shaw and J. P. Jeffries, *J. Pharm. Pharmacol.* 3, 823-8 (1951).

CHAPTER 3

AMINO ACIDS[1]

Amino acids are heterogeneous in that, except from having a carboxyl group and an amino group, they may be as short chain as glycine or a much longer chain, or they may be entirely aliphatic or contain an aromatic or heterocyclic nucleus. This heterogeneity naturally permits of a host of methods, depending on the structure, even to nitration of the aromatic nucleus, reduction to an aromatic amine, and coupling. Because their determination often depends on their structural sulfur; cystine, cysteine, and methionine are excluded from this chapter and classed with sulfur compounds (Vol. III, pages 478-489, 497-499).

General reactions with triketohydrindene hydrate and enzyme, with 2,4-dinitrophenylhydrazine, and with sodium-2-naphthoquinone-4-sulfonate, applicable with greater or lesser accuracy to all α-amino acids in the absence of others, are given at the beginning of the chapter before taking up specific amino acids.

It has seemed desirable to place these in order of increasing chain length of the aliphatic acid. For somewhat greater clarity in giving the chemical structure, the substituent is always given first and numbers have been used in place of α, β, γ, δ, etc. Since the Greek letters or arbitrary names are more conventionally used in this particular field, that has not been carried into the text. Thus tyrosine would be 2-(*p*-hydroxyphenyl-1-aminopropionic acid rather than β-(*p*-hydroxyphenyl) alanine.

α-Amino Acids in General

Substantially all α-amino acids are decarboxylated and diaminated by triketohydrindene hydrate, ninhydrin, to give a blue coloration in alkaline solution.[2] No aldehydes are formed from β or ϵ amino acids.

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[1] See Chapter 1, Volume III, for details of organization, condensations, etc.

[2] S. Ruhemann, *J. Chem. Soc.* **97**, 2025-31 (1910); *Ibid.* **99**, 792-800 (1911); Emil Abderhalden and Hubert Schmidt, *Z. physiol. Chem.* **72**, 37-44 (1911); *Ibid.* **85**, 143-7 (1913); Victor J. Harding and Reginald M. MacLean, *J. Biol. Chem.* **20**, 217-30 (1915); *Ibid.* **25**, 337-50 (1916); Stanford Moore and William H. Stein, *Ibid.* **176**, 367-88 (1948); Radwan Moubasher and William Ibrahim Awad, *Ibid.* **179**, 915-20 (1949); Theodore B. Schwartz and Frank L. Engel, *Ibid.* **184**, 197-202 (1950); L. Fowden, *Biochem. J.* **48**, 327-33 (1951); Walter Troll and R. Keith Cannan, *J. Biol. Chem.* **200**, 803-11 (1953).

The reaction is also given by peptides, proteins, and other types of substances having the group $-COCH(NH_2)-$. The ketone of nihydrin is correspondingly reduced to a carbinol group. As to mechanism, under some conditions ammonia is liberated in free form, under others it condenses with the reagent to form diketohydrindylidenediketohydrindamine. In phenol-pyridine containing 5 per cent of water, color development at 570 mμ is complete in 20 minutes.

Oxidation must be avoided, conveniently by addition of a reduction product of ninhydrin, hydrinanthin, or production of that reduction product in the technic by adding stannous chloride. The color per mole is not uniform as shown by Table 9. It is usually approximated. By absorption spectra all α-amino acids except cysteine give the same blue major end product.

There is no interference by adenine, *p*-aminobenzoic acid, diethylbarbituric acid, glucose, or uric acid. Values of less than 5 per cent of the molar reaction of α-amino acids by creatine, creatinine, dibenzylamine, glycine anhydride, and urea suggest that their interference will not be significant. Glucosamine and ammonia gives the same degree of reaction as an α-amino acid. Some related compounds are shown in the table. An alternative is to heat the sample solution with pyridine, ascorbic acid and ninhydrin, oxidize the red color to purple with air, and read at 570 mμ.[3]

By deamination in the pH range 1-3, quantitative production of Δ^2,Δ^3-2,2*N*-bisendandeone is accomplished. Then at pH 11 or higher the ammonia produced is aerated out and determined by Nessler's reagent.[4] Anomalous results are more from peculiar behavior of the amino acids than of the reagent. Thus sources of error are (1) incomplete yield from aspartic acid and tryptophane, (2) oxidation with incomplete release of ammonia from proline and hydroxyproline, (3) release of δ-guanadino nitrogen from arginine, and (4) release of ε-amino nitrogen from lysine. The nitrogen not recovered from tryptophane is no longer present as amino acid nitrogen, which is to say the reaction takes two courses[5] with that particular amino acid. Cystine reacts quantitatively at pH 1 but not at pH 2.

[3] Mosaharu Yamagishi and Toshiko Yoshida, *J. Pharm Soc. Japan* **73**, 675-6 (1953).

[4] Frank S. Schlenker, *Plant Physiol.* **18**, 141-9 (1943); Albert E. Sobel, Albert Hirschman, and Lottie Besman, *J. Biol. Chem.* **161**, 99-103 (1945); Frank S. Schlenker, *Ind. Eng. Chem., Anal. Ed.* **19**, 471-4 (1947).

[5] Douglas A. MacFadyen, *J. Biol. Chem.* **153**, 507-13 (1944).

TABLE 9. DEGREE OF COLOR DEVELOPMENT WITH NINHYDRIN

Common Name	*Chemical Name*	*Molar Degree of Color Development*
α-Alanine	α-aminopropionic acid	1.01
Arginine	guanidine-α-aminovaleric acid	1.00
Aspartic acid	aminosuccinic acid	0.88
Citrulline	α-amino-δ-carbamidovaleric acid	1.03
Cysteine	α-amino-β-mercaptopropionic acid c	0.15
Half-cystine		0.54
Glutamic acid	α-aminoglutaric acid	1.05
Glycine	aminoacetic acid	1.01
Ethyl glycine		1.00
Glycyl glycine		0.89
Leucyl glycine		0.92
Phenylalanyl glycine		0.97
Histidine	β-imidazolyl-α-alanine	1.04
Leucine	α-aminoisocaproic acid	1.00
Isoleucine	α-aminocaproic acid	1.00
Glycyl leucine		1.05
Lysine	α,ε-diaminocaproic acid	1.12
Methionine	α-amino-γ-methylthiolbutyric acid	1.00
Phenylalanine	α-amino-β-phenylpropionic acid	0.88
Ethyl phenylalanine		0.98
Glycyl phenylalanine		1.04
Serine	α-amino-β-hydroxypropionic acid	0.94
Tryphophane	β-indolyl-α-alanine c	0.72
Tyrosine	β-(*p*-hydroxyphenyl)-alanine	0.88
Glycyltyrosine		0.88
Threonine	α-amino-β-hydroxybutryic acid	0.92
Valine	α-aminoisovaleric acid	1.02
Asparagin	α-aminosuccinic acid amide	0.94
Glutamine	glutamic acid amide	0.99
Glutathione		0.76
Histamine	β-amidazolyl-4-ethylamine	0.65
Hydroxyproline		0.03
Proline	pyrrolidine-2-carboxylic acid	0.05
Sarcosine	*N*-methylglycine c	0.64
Taurine	β-aminoethylsulfonic acid	0.97
Tyramine	Phenylethylamine	0.64

Another method for total α-amino acids is to deaminate with enzyme and develop with 2,4-dinitrophenylhydrazine, reading in strongly alkaline solution. Aldehydes produced by ninhydrin are also reacted with this reagent.[6]

Sodium-2-naphthoquinone-4-sulfonate is a general reagent for amino

[6] F. Turba and E. V. Schrader Beielstein, *Naturwissenschaften* 34, 57 S (1947)

acids.[7] The depth of the orange-red color complex increases in line with inductive effects of the radical attached so that the extinction for lysine approximates double that for arginine. It follows that it is not suitable for estimation of total amino acids. The color is due to formation of a 2-hydroxy-1,4-naphthoquinone imine with CHRCOOH attached to the nitrogen. The R is the amino acid residue. The method is appropriate for estimation of isolated amino acids. Heating the reaction mixture hastens the reaction.[8]

Amino acids, in the absence of other substances oxidizable by sodium hypochlorite, react with 2 moles of oxidizing agent.[9] Excess reacts with Fast Green FCF of which the excess is determined. The latter only reacts with hypochlorite at pH 1 or below. Accuracy to ±2 per cent is obtainable but other oxidizable groups must be absent. Thus the SH group of cysteine will react.

Another technic is to precipitate the copper salt of the amino acid and determine copper with diethyl dithiocarbamate.[10] In another version the copper amino acid salt is converted to the copper salt of alanine.[11] The principle is that an excess of one amino acid will convert the copper salt of any amino acid largely to the salt of that acid by mass action. Alanine is appropriate because it has an intermediate color intensity. The copper salt of the amino acid is formed from the equivalent copper phosphate. This is then read as copper alaninate which for all amino acids except histidine hydrochloride gives a value between 98.2 and 102.4 per cent of the theoretical value.

Procedure—*By ninhydrin.* Prepare a buffer for pH 5 containing 2.008 grams of citric acid monohydrate and 200 ml. of 4 per cent sodium hydroxide. Dilute this to 500 ml. and check as pH ±0.1. As reagent add 0.8 gram of stannous chloride dihydrate to 500 ml. of this citrate

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[7] Otto Folin, *J. Biol. Chem.* **51**, 377-94 (1922); Melville Sahyun and M. Goodell, *J. Lab. Clin. Med.* **24**, 548-53 (1939); Teturo Hori, *J. Biochem.* (Japan) **32**, 121-30 (1940); D. T. Englis and H. A. Fiess, *Ind. Eng. Chem.* **34**, 864 (1942); Elizabeth G. Frame, Jane A. Russell, and Alfred E. Wilhelmi, *J. Biol. Chem.* **149**, 255-70 (1943); Kathryn K. Krauel, *J. Lab. Clin. Med.* **29**, 222-7 (1944); Jane A. Russell, *J. Biol. Chem.* **156**, 467-8 (1944); Walter S. Hoffman, *Am. J. Clin. Path., Tech. Sect.* **9**, 57-60 (1945); N. Howell Furman, George H. Morrison, and Arthur F. Wagner, *Anal. Chem.* **22**, 1561-2 (1950).

[8] Melville Sayhun, *J. Lab. Clin. Med.* **24**, 548-53 (1939).

[9] H. T. Gordon, *Anal. Chem.* **23**, 1853-8 (1951).

[10] A. J. Woiwood, *Biochem. J.* **45**, 412-17 (1949).

[11] Joseph R. Spies and Dorris C. Chambers, *J. Biol. Chem.* **191**, 787-97 (1951).

buffer. Add this to 20 grams of recrystallized ninhydrin in 500 ml. of methyl Cellosolve and mix. This keeps under nitrogen for a month.

Neutralize a sample if acid. To 2 ml. of reagent add 0.5 ml. of a solution containing around 0.002 mole of the amino acid. Shake and heat in boiling water for 20 minutes. Add 5 ml. of 50 per cent *n*-propanol with mixing. Read after 15 minutes at 570 mμ against a reagent blank. For application to isolated amino acids, Table 10 shows the wave length of maximum absorption and limits applicability of Beer's law.[12]

TABLE 10. WAVE LENGTH OF COLOR REACTION OF SPECIFIC PROTEINS WITH NINHYDRIN

Amino Acid	*Wave length of Maximum Absorption* (mμ)	*Limits Over Which Beer's Law Holds (micrograms per ml.)*
Glycine	555	10-80
Alanine	550	10-80
Phenylalanine	530	10-140
Tryptophan	550	20-200
Threonine	550	20-130
Valine	560	10-100
Norvaline	560	10-70
Arginine	560	20-100
Isoleucine	565	20-125
Leucine	555	10-100
Asparagine	550	20-180
Lysine	545	5-50
Methionine	560	10-100

Alternatively form a reagent by diluting 2 ml. of 0.065 per cent potassium cyanide solution with pyridine to 100 ml., the pyridine having been shaken with 1 per cent of Permutit for 20 minutes to free it from ammonia. Add 1 ml. to 0.4-0.5 ml. of amino acid solution and follow with 1 ml. of 1:4 ethanol-phenol, also deammoniated with Permutit. Heat in boiling water and when it reaches the temperature of the bath, add 0.2 ml. of 5 per cent ninhydrin in absolute ethanol. Stopper for 3-5 minutes while heating, cool, and dilute to 60 ml. with 60 per cent ethanol. Read at 570 mμ against a reagent blank.

[12] William H. Fitzpatrick, *Science* 109, 469 (1949).

As ammonia. Mix 1 ml. of sample solution containing 0.05-0.15 mg. of α-acid nitrogen with 1 ml. of 56 per cent phosphoric acid as a buffer for pH 1. Mix with 1 ml. of 1.5 per cent ninhydrin solution and stopper loosely. After 1 hour in boiling water, cool and add 5-10 ml. of water.

To isolate the ammonia by aeration add a drop of a 1 per cent aqueous dispersion of phenolphthalein, 1 ml. of 5 per cent paraffin solution in toluene, and sufficient saturated aqueous sodium hydroxide to make the solution strongly alkaline. Aerate into 10 ml. of 1:3600 sulfuric acid at 55-60° for 0.5 hour.

Aspartic acid and glutamic acid at room temperature give 98.5 and 94 per cent recovery after ninhydrin reaction. Dilute the contents of the receiving flask to nearly 45 ml., and add 5 ml. of Nessler's reagent (page 181). Dilute to 50 ml. and read at 410 mμ.[13]

By 2,4-dinitrophenylhydrazine. Adjust a 2-ml. sample to pH 8 and add 2 ml. of *d*-deaminase in 0.45 per cent sodium pyrophosphate solution. Flush the flask with oxygen and stopper tightly. Incubate at 38° until reaction ceases and add 1 ml. of 20 per cent trichloroacetic acid solution. Dilute to 10 ml. and filter out the flocculate. Mix 1 ml. of filtrate, more if needed to develop adequate color, with 1 ml. of half-saturated 2,4-dinitrophenylhydrazine in 1:10 hydrochloric acid. After 10 minutes add 10 ml. of 8 per cent sodium hydroxide and dilute to 25 ml. Read at 520 mμ and subtract a reading of the sample before incubation.

By sodium-2-naphthoquinone-4-sulfonate. Dilute a sample containing 0.01-0.03 mg. of amino nitrogen to 5 ml. and add 1 drop of 0.25 per cent alcoholic phenolphthalein solution. Add 0.4 per cent sodium hydroxide solution dropwise until a permanent pink color is established. Add 1 ml. of 1 per cent borax solution as a pH buffer. Add 1 ml. of freshly prepared 0.5 per cent 1,2-naphthoquinone-4-sulfonate solution and mix. Heat in boiling water for 10 minutes, then cool in cold water for 5 minutes. To acidify the mixture add 1 ml. of a solution composed of 3 parts of 1:7 hydrochloric acid, 1 part of acetic acid, and 4 parts of 1:50 commercial formaldehyde, then mix. To decolorize the excess naphthoquinone add 1 ml. of 2.5 per cent sodium thiosulfate solution. Dilute to 15 ml. with water, mix, and allow to stand for 30 minutes. Read at 480 or 490 mμ against a reagent blank.

By hypochlorite and Fast Green FCF. As hypochlorite bubble

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[13] For more details of problems involved in this estimation see Vol. II, pp. 814 to 818.

chlorine gas through cold 2 per cent sodium hydroxide solution for an hour. Store at 5° until used. For standardization mix 0.1 ml. with 1 ml. of water and add exactly 0.15 ml. of 0.1 per cent Fast Green FCF in 1:17 sulfuric acid. After 30 seconds add 1 ml. of sulfate buffer (page 374) and dilute to 10 ml. Read against a water blank.

Mix a sample containing 0.02 mg. of glycine or the equivalent of a high molecular weight acid with 1 ml. of water and add 0.095 ml. of standardized hypochlorite reagent. Mix and after 10 minutes add 0.15 ml. of the dye solution. After 30 seconds add 1 ml. of the sulfate buffer used in standardization. Dilute to 10 ml., read against a water blank, and calculate on the basis that one mole of amino acid reacts with two moles of hypochlorite.

As copper alaninate. To 40 ml. of 6.85 per cent trisodium phosphate solution add 20 ml. of 2.8 per cent cupric chloride dihydrate solution. Swirl and then centrifuge for 5 minutes. Wash twice by resuspension in 60 ml. of 1 per cent sodium tetraborate solution and centrifuging. Suspend the precipitate in 100 ml. of the borate solution and add 6 grams of sodium chloride. This suspension gives the maximum color when 4 days old.

To 5 ml. of sample solution at pH 7 add 5 ml. of the copper phosphate suspension. Shake occasionally for 5 minutes and centrifuge. Decant the blue solution and add 200 mg. of alanine. When the alanine is dissolved read at 620 mμ against a reagent blank.

Aminoacetic Acid, Glycine

Glycine is decarboxylated, deaminated, and converted to the aldehyde with one less carbon by ninhydrin. The formaldehyde so formed is measured by the color from reaction with chromotropic acid.[14] The method will detect 0.5 microgram of glycine. It is essential that not over 3 micrograms of glycine nitrogen be present [15] because at higher concentrations the losses are excessive.[16] Rapid distillation is also essential. The reaction between formaldehyde and chromotropic acid is specific.

Glyceraldehyde, arabinose, fructose, and cane sugar react with the

[14] Benjamin Alexander, Greta Landwehr, and Arnold M. Seligman, *J. Biol. Chem.* **160**, 51-9 (1945).

[15] Halvor N. Christensen, Thomas R. Riggs, and Nancy E. Ray, *Anal. Chem.* **23**, 1521-2 (1951).

[16] R. Krueger, *Helv. Chim. Acta* **32**, 238-51 (1949).

reagent to give a yellow color and do not interfere. Furfural in high concentrations can interfere because it gives a pink color, as does formaldehyde. Glucosamine can be hydrolyzed to give formaldehyde [17] but, under the conditions of this method, it does not break down. No formaldehyde is given by aspartic acid, alanine, tyrosine, lysine, leucine, isoleucine, norleucine, trytophan, ornithene, threonine, phenylalanine, proline, hydroxyproline, methionine, cystine, cysteine, homocysteine, valine, norvaline, diiodotyrosine, arginine, serine, β-alanine, glutamic acid, α-aminoisobutyric acid, β-amino-n-butyric acid, or benzoylalanine. Alanylglycine interferes if there are 5 equivalents for 1 part of glycine. Unless blood filtrates are distilled promptly after addition of ninhydrin, results will be low due to reaction of the aldehyde with amino acids.

The green color developed with *o*-phthalyldialdehyde is chloroform-soluble and suitable for colorimetric estimation of glycine.[18] Alanine and asparagine give a red color which is not extractable with chloroform. These as well as argenine react so weakly that they do not interfere. Cystine gives a fairly strong color, which is also not extractable. Tryptophan and ammonium chloride give a dark brown-green in the aqueous layer and a blue in the chloroform layer. Argenine, histidine, and histamine also interfere.

No interference occurs with valine, leucine, tyrosine, serine, phenylserine, aspartic acid, glutamic acid, lysine, proline, hydroxyproline, betaine, trigonellin, nicotinic acid, choline, dimethylamine, diethylamine, dibutylamine, trimethylamine, triethylamine, or tripropylamine. Large concentrations of methylamine, propylamine, butylamine, hexylamine, heptylamine, and phenol give yellow to brown colors. Indol gives a yellow color. The color intensity is directly related to the amount of reagent used. The yellow or brown color of protein hydrolyzates is not extracted by chloroform and therefore does not interfere. The reagent must be prepared.[19]

The color produced by the reaction of glycine with alloxan is pink in 15 minutes and reddish-violet after 4 hours. On addition of water and neutralization with sodium hydroxide solution, it becomes violet-

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[17] M. J. Boyd and M. A. Logan, *J. Biol. Chem.* **146**, 279 (1942).

[18] Walther Zimmermann. *Z. physiol. Chem.* **189**, 4-6 (1930); G. Klein and H. Linser, *Ibid.* **205**, 251-8 (1932); A. R. Patton, *J. Biol. Chem.* **108**, 267-72 (1935); Karl Brecht and Gerolf Grundmann, *Biochem. Z.* **302**, 42-9 (1939).

[19] W. M. Sandstrom and H. A. Lillevik, *Ind. Eng. Chem., Anal. Ed.* **13**, 781 (1941).

red. Addition of zinc acetate converts it to a yellowish-orange.[20] Evaporation must be at room temperature rather than on the hot plate. Concentrated ammonium acetate gives a pink color with the reagent. Interfering substances in order of importance are alanine, tryptophan, phenylalanine, and leucine. Creatinine gives the reaction, but creatine does not. Hippuric acid, glycocholic acid, and taurocholic acid do not interfere.

Samples—*Blood.* Use a tungstic-acid filtrate for development with chromotropic acid.

Urine. Use a 5-ml. aliquot of a 1:50 dilution for development with chromotropic acid.

Protein. Reflux 3 grams of protein with 50 ml. of concentrated hydrochloric acid until the protein dissolves completely. As soon as this happens add 1 ml. of benzaldehyde and continue to reflux for 24 hours. Distil *in vacuo* from a water bath at 65° until only a paste remains. This removes all of the excess benzaldehyde and nearly all of the free hydrochloric acid. Add 100 ml. of water to the residue and a few ml. of butanol to prevent foaming. Add excess of sodium bicarbonate and distil *in vacuo* at 40-50° under 30 mm. to remove ammonia. Sodium chloride will begin to separate when about 20 ml. of solution remain.

Filter the solution quickly with suction and wash the precipitate of carbonate and chloride with a few ml. of 70 per cent ethanol. Add 1:1 hydrochloric acid to the filtrate until it is neutral to litmus. If the solution remains alkaline until the buffer is added, the color developed will be lessened. If it remains acid after adding the buffer, no color will develop. Evaporate *in vacuo* to about 10 ml. Filter with suction and wash the precipitate of sodium chloride with a few ml. of 70 per cent ethanol. Dilute the filtrate to 100 ml. for development of aliquots with *o*-tetrabromo-*o*-xylene.

Procedure—*By chromotropic acid.* Add 3 ml. of sample solution containing not over 1.5 micrograms of glycine nitrogen to 2 ml. of a phosphate buffer containing 3.5 grams of tripotassium phosphate in 100 ml. of 20 per cent monopotassium phosphate solution, 1 ml. of 1 per cent ninhydrin solution, and a glass bead in an all-glass Stotz still (Vol III, page 262). Distil rapidly over a period of 3-4 minutes, nearly to dryness and cool the distilling flask to room temperature in a water

[20] Georges Deniges, *Bull. soc. pharm. Bordeaux* 73, 161-8 (1935).

bath. Add 2 ml. of water and continue the distillation to dryness. Dilute to 10 ml. and mix thoroughly.

Cool and agitate a 5-ml. aliquot in an ice bath, while slowly adding 4 ml. of concentrated sulfuric acid. When the reaction mixture is at room temperature, add 3 drops of 5 per cent chromotropic acid solution. Shake, stopper lightly, and heat at 100° for 30 minutes. Cool and read at 565 mμ against a reagent blank.

By o-phthalyldialdehyde. For preparation of the reagent, fit a 1-liter three-necked balloon flask with a calcium chloride drying tube, a dropping funnel, and a stirrer. Cool the flask in an ice bath while 150 ml. of acetic anhydride, 10 grams of *o*-xylene, and 8 ml. of concentrated sulfuric acid are introduced. Dissolve 26 grams of chromium trioxide in a mixture of 50 ml. of acetic anhydride and 60 ml. of glacial acetic acid. Add this solution drop by drop from the funnel with stirring. Keep the reaction flask in the ice bath for 4 or 5 hours, during which time stir the mixture continuously. Pour the contents into a 1-liter beaker one-fourth filled with cracked ice and place in an icebox overnight. A yellow oily scum collects on the surface. Extract the whole several times with ether and wash the combined ether extracts with water to remove the last traces of chromous compounds. Dry over anhydrous sodium sulfate. Distil the ether on a steam bath and remove the last by a vacuum. Treat the residue, consisting of acetic acid plus phthaldihyde tetra-acetate, with 50 ml. of 10 per cent sulfuric acid and steam-distil as long as the distillate gives a blue color upon adding a drop of ammonium hydroxide and acidifying with acetic acid. Store the approximately 500 ml. of reagent in a dark bottle.

To 5 ml. of the sample solution add 2 ml. of buffer for pH 8 (Vol. I, page 176). Immediately add 5 ml. of reagent, mix well, and let stand for 2 minutes. Add 5 ml. of a fresh, cold mixture of 60 ml. of ethanol and 10 ml. of concentrated sulfuric acid. Mix and add 10 ml. of chloroform. Stopper, shake vigorously for one-half minute, and let the chloroform layer settle. Pipet out 5 ml. of the green bottom layer, add 1 ml. of ethanol, and stir to remove all turbidity. Read at 720 mμ against a reagent blank.

Phenylaminoacetic Acid

Phenylaminoacetic acid is decomposed by peri-naphthindan-2,3,4-trione to give benzaldehyde which is estimated by reaction with alkaline salicylic aldehyde. For details see valine (page 152).

Benzoyl Glycine, Benzoylaminoacetic Acid, Hippuric Acid

Sodium hypobromite reacts with hippuric acid to give a cochineal-red substance.[21] Benzoic acid does not interfere. The reagent can be replaced by sodium hypochlorite, but the color is yellowish and the reaction less sensitive.

The method for nitration of benzoic acid, reduction with titanous chloride to the amine, diazotizing, and coupling (Vol. III, page 404) is equally applicable to hippuric acid in the absence of benzoic acid.

Procedure—Prepare the reagent by adding 20 ml. of water and 1 ml. of bromine to 10 ml. of a 30 per cent sodium hydroxide solution. Add 2 ml. of this reagent to 5 ml. of test solution at 100°. Stir for 30 seconds, chill, and add 1 ml. of chloroform. Shake to extract the color, remove the solvent layer, and read against a chloroform blank.

1-Aminopropionic Acid, Alanine

One method of estimation of alanine is by conversion to lactic acid with nitrous acid[22] and determination in that form.[23] As a convenient device the lactic acid is oxidized to acetaldehyde for determination by *p*-hydroxybiphenyl.[24] This method requires simultaneous determination of threonine since it too yields acetaldehyde on oxidation. The steps are conveniently coalesced by converting alanine to acetaldehyde by ninhydrin for the latter form of estimation.[25,26] To minimize interference, the acetaldehyde is quantitatively distilled from the reaction mixture.

The method is sensitive to 0.04 mg. per ml. of whole blood. For determinations on blood levels lower than this, use a more concentrated blood filtrate, use a larger volume of blood filtrate, aerate the acetaldehyde into a smaller volume of bisulfite solution, or redistil the bisulfite-

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[21] Georges Denigès, *Compt. rend.*, 209, 972-4 (1939); *Ibid.* 107, 163 (1888).

[22] Otto Fürth, Rudolf Scholl and Heinz Herrmann, *Biochem. Z.* 251, 404-18 (1932).

[23] Theodore E. Friedemann, Margherita Cotonio, and Philip A. Schaffer, *J. Biol. Chem.* 73, 335-58 (1927).

[24] R. J. Block, D. Bolling, and M. Webb, *J. Biol. Chem.* 133, *Proc. Am. Soc. Biol. Chem.*, XIV (1940).

[25] Aturri I. Virtanen, T. Laine, and Taini Toivonen, *Z. physiol. Chem.* 266, 193-204 (1940); Benjamin Alexander and Arnold M. Seligman, *J. Biol. Chem.* 159, 9-19 (1945); B. F. Folkes, *Analyst*, 78, 496-8 (1953).

[26] For a general discussion of this reaction, see page 104.

acetaldehyde solution.[27] To get complete conversion of 0.02-0.2 mg. of alanine to acetaldehyde, it must be boiled for 1 hour with 10 mg. of ninhydrin or autoclaved at 125° for 10 minutes.

There is no interference from pyruvic acid, alanylglycine, benzoylalanine, β-alanine, threonine, serine, phenylalanine, glutamic acid, tryptophan, cystine, cysteine, valine, ornithine, lysine, α-amino-*n*-butyric acid, methionine, arginine, isoleucine, tyrosine, diiodityrosine, α-aminoisobutyric acid, proline, and hydroxyproline. Aspartic acid reacts with ninhydrin to form acetaldehyde but the amount formed can be controlled by the pH at which the reaction takes place. Thus at pH 5.5-5.6 no more than 4 per cent of aspartic acid is converted to acetaldehyde and no interference in aeration occurs.

Interfering aldehydes formed by the reaction are formaldehyde from glycine, isovaleraldehyde from leucine, *n*-valeraldehyde from norleucine, and *n*-butyraldehyde from norvaline. Formaldehyde gives a blue-green color.[28] Isovaleraldehyde and *n*-valeraldehyde give a rose-pink color. However, *n*-butyraldehyde gives a violet color which is difficult to distinguish from acetaldehyde.

The difficulty arising from formaldehyde is avoided by separating it from acetaldehyde by prolonged aeration of the refluxing mixture.[29] The amount of color which leucine gives varies with the temperature of the sulfuric acid mixture when the *p*-hydroxybiphenyl is added. An increase in the temperature at which it is reacted with the aldehyde gives a decrease in the intensity of the color. Interference from norleucine and norvaline is difficult to circumvent, but by raising the temperature of the aldehyde-sulfuric acid mixture to 37°, their interference is reduced. Fortunately norleucine is rarely present in proteins.

Oxidation of alanine, valine, and leucine with sodium hypochlorite gives volatile aldehydes [30] subject to the same reactions for color development. Alanine in protein hydrolyzates is converted to acetaldehyde by permanganate oxidation and the latter determined with sodium nitroprusside and piperazine by reading at 560 mμ. Serine and aspartic acid give the same reaction but can be prevented from interfering by addition of mercuric acetate during the oxidation process.[31] The method has been

[27] Elmer Stotz, *J. Biol. Chem.* **148**, 585 (1943).

[28] S. B. Barker and William H. Summerson, *Ibid.* **138**, 535-54 (1941).

[29] L. A. Shinn and B. A. Nicolet, *Ibid.* **138**, 91 (1941).

[30] E. Aubel and J. Asselineau, *Bull. soc. chim. France* **1947**, 114-15.

[31] C. Fromageot and P. Heitz, *Mikrochim. Acta* **3**, 52-67 (1938).

applied to edestin, casein, and the protein of the yellow enzyme.[32] Propionaldehyde gives the reaction but the color is only about 2 per cent of that with acetaldehyde. For 2 mg. or more of alanine the method is accurate to about ±6 per cent.

Alanine is decomposed by *peri*-naphthindan-2,3,4-trione hydrate to acetaldehyde which is estimated with salicylic aldehyde and alkali. For details see valine (page 152).

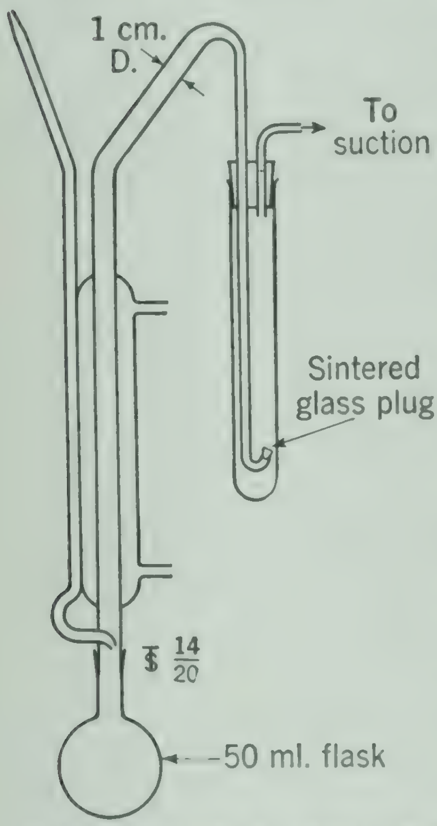

Fig. 7. Apparatus for aeration of acetaldehyde from refluxing reaction mixture. The air inlet tube is placed in contact with the water-cooled condenser to condense any steam that may back up during bumping. The sintered glass plug may be replaced by a capillary opening

Sample—*Blood.* Use a tungstic acid filtrate. Alternatively deproteinize with picric acid.[33] Develop with *p*-hydroxybiphenyl after conversion to acetaldehyde.

Proteins. Hydrolyze and convert the amino acids to hydroxy acids according to the method for isoleucine (page 159) to "adjust the volume to roughly 30 ml." If substances such as carbohydrates are present which would interfere with determination of the lactic acid formed from the alanine, remove these before diluting to volume, by adding 10 ml. of a 20 per cent copper sulfate solution, and 10 ml. of a 20 per cent suspension of calcium hydroxide. Shake and dilute to 100 ml. The solution so obtained is appropriate for conversion to acetaldehyde and development with sodium nitroprusside-piperazine reagent.

Procedure—*By p-hydroxybiphenyl.* To 5 ml. of sample in the flask of the apparatus of Figure 7 add 1 ml. of 1 per cent ninhydrin solution and 2 ml. of phosphate buffer solution prepared by adding 3.5 grams of tripotassium phosphate to 100 ml. of a 20 per cent solution of pri-

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[32] Pierre Desnuelle, *Enzymologia* 5, 37-43 (1938).
[33] O. Wiss, *Helv. Chim. Acta* 31, 22-6 (1948).

mary potassium phosphate. Add a glass bead and attach the flask to the aeration still which has on its receiving end a test tube containing 8 ml. of 1 per cent sodium bisulfite solution. Immerse this tube in an ice bath. Apply a gentle suction and reflux the reaction mixture for 75 minutes. Keep a steady stream of air flowing through the apparatus.

Remove the test tube containing sodium bisulfite and acetaldehyde and dilute to 10 ml. Complete as for lactic acid by the same reagent (Vol. III, pages 348-9) using 1 ml. and starting at "To the aliquot containing . . .." Calibration curves prepared directly from lactic acid cannot be used.

By sodium nitroprusside-piperazine reagent. To an aliquot in a distilling flask corresponding to 2 mg. or more of alanine, add 10 ml. of 6.4 per cent phosphoric acid and enough 10 per cent manganous sulfate solution to give a 2 per cent solution of the latter. For example, add 10 ml. of manganous sulfate solution to a 50-ml. aliquot. Add a pinch of talc and a one-tenth volume of a 5 per cent mercuric acetate solution containing 1 per cent of acetic acid, in order to prevent interference from the serine and aspartic acid originally present. Connect the flask for steam-distillation and add 0.632 per cent potassium permanganate solution for 10 minutes, at the rate of a drop a second. Continue boiling for 10 minutes after this amount of permanganate has been added, distilling into a 1 per cent solution of sodium bisulfite. Add 10 ml. of a saturated solution of sodium bicarbonate and destroy the excess bisulfite by accurate titration with standard iodine solution. Suppress any trace of blue color by addition of a drop of 2.5 per cent sodium thiosulfate solution and dilute to 100 ml. for use as a sample.

To 5 ml. of the sample solution add 5 ml. of saturated aqueous piperazine hydrate and 2.5 ml. of 4 per cent sodium nitroprusside solution. Dilute to 25 ml. and read at 570 mμ against a reagent blank.

2-Hydroxy-1-aminopropionic Acid, Serine

Oxidation of serine by periodate yields formaldehyde quantitatively.[34] In the presence of arsenite, to prevent decomposition of periodic acid, the formaldehyde is distilled and determined quantitatively with chromotropic acid. Since that reagent is specific for formaldehyde, there is no interference from acetaldehyde, resulting from oxidation of threo-

[34] Ben H. Nicolet and L. A. Shinn, *J. Am. Chem. Soc.* **61**, 1615 (1939); *J. Biol. Chem.* **139**, 687-92 (1941); P. Desnuelle and S. Antonio, *Biochim. et Biophys. Acta* **1**, 50-60 (1947).

nine, and higher homologs. On long standing, cysteine is spontaneously converted in part to serine. Sugars must be absent. Lactose does not interfere. Glucosamine gives about 10 per cent of the value of serine. The reaction is sensitive to 0.05-0.1 mg. of formaldehyde and the method has an error of ±2 per cent.[35] An alternative is to determine serine, aspartic acid, and alanine together, subtract alanine as determined separately, and so get the sum of serine and aspartic acid.

Serine and threonine are also determined simultaneously by the first being oxidized to formaldehyde and the second to acetaldehyde, the two aldehydes being separated thereafter.[36] The formaldehyde is then determined by chromotropic acid and acetaldehyde by *p*-hydroxybiphenyl. Accuracy of each is in the range 6-9 per cent. Hydroxylysine interferes with determination of serine.

Sample—*Protein for serine.* Reflux 1 gram of protein with 40 ml. of water and 50 ml. of concentrated hydrochloric acid for 24 hours. Cool and dilute to 100 ml. If humin is present, centrifuge before aliquoting. While acid hydrolysis destroys serine, the loss is only about 1 per cent in 24 hours.

Protein for serine and threonine. Heat 0.1 gram of protein with 5 ml. of 1:1 hydrochloric acid in a wax bath at 120-125° for 6 hours. Neutralize the hydrolyzate with 20 per cent sodium hydroxide solution dropwise with stirring until the pH is about 3.5. Dilute to 50 ml. with water.

Procedure—*Serine by distillation.* Add 3 drops of methyl red to a neutral solution containing 1-5 mg. of serine. Follow with 4 ml. of 25 per cent potassium arsenite and 2.5-2.8 ml. of 11.4 per cent periodic acid. Add the last 0.5 ml. of acid dropwise with continuous gentle shaking. Stop adding periodic acid when the mixture is acid to methyl red. Add a pinch of talc and enough water to make the final volume 70 ml. Start the distillation, having the end of the condenser dipping into a receiver containing about 10 ml. of water. Distil all but about 5 ml. of the mixture. Swirl the contents of the flask during the distillation to wash down the iodate which spatters and becomes dry on the sides of the flask. Dilute the distillate to a volume of 100 ml.

As chromotropic acid reagent dissolve 0.9 gram in 25 ml. of water.

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35 M. John Boyd and Milan A. Logan, *J. Biol. Chem.* **146**, 279-87 (1942); Raymond Michel and Mireille Bozzi Tichadon, *Bull. soc. chim. biol.* **29**, 883-5 (1947).

36 B. A. Neidig and W. C. Hess, *Anal. Chem.* **24**, 1627-8 (1952).

Add about 50 mg. of stannous chloride, shake, and centrifuge. By avoiding air exposure, this keeps for 2 days.

Add 0.5 ml. of the chromotropic acid reagent to a quantity of distillate containing 0.05-0.1 mg. of formaldehyde. Dilute to 17 ml. and cool in an ice bath. Add 10 ml. of concentrated sulfuric acid with gentle shaking during a 40-45 second period. Cool and pour concentrated sulfuric acid down the center of the tube till the volume is 50 ml. The temperature of the solution will rise to about 80°. Heat for 10 minutes in a boiling water bath, cool to room temperature, and read at 565 mμ against a reagent blank.

Serine without distillation. Dilute a solution containing 0.2-1.4 mg. of serine without interfering amino acids, to 3 ml. and adjust to approximately pH 7. Add 0.7 ml. of a 15 per cent solution of sodium periodate. Let stand at 27° for 20 minutes, cool in ice, and add 0.4 ml. of saturated potassium nitrate solution.

Dilute an aliquot of this solution to 10 ml. and add 2 ml. of a freshly prepared 1 per cent solution of phenylhydrazine hydrochloride. Let stand at about 27° for 20 minutes, cool in ice water, and add 1 ml. of a freshly prepared 2 per cent solution of potassium ferricyanide. Let stand in ice water for 20 minutes and add carefully with stirring 5 ml. of chilled concentrated hydrochloric acid. Read at 530 mμ against a reagent blank.

Serine and threonine by aeration. Prepare an oxidation and absorption train of a sulfuric acid drying flask, three 20 x 2.5 cm. test tubes, and a suction trap prior to a filter pump. Add 5 ml. of sample containing 0.02-0.1 mg. of serine and threonine. Cool the second and third tubes containing 5 ml. each of 2 per cent sodium bisulfite solution with an ice bath. To the sample add 15 ml. of buffer for pH 8 containing 25 ml. of 1.5 per cent potassium chloride solution and 1.99 ml. of 0.8 per cent sodium hydroxide solution per 100 ml. Add 1 ml. of 5 per cent periodic acid solution and 2 ml. of 0.8 per cent sodium hydroxide solution. Pass a fairly rapid stream of air through the system for 1 hour. Combine the contents of the sodium bisulfite tubes and after 1 hour use for determination of acetaldehyde. Reserve the contents of the sample tube for determination of formaldehyde.

Acetaldehyde from threonine. To a 1-ml. aliquot of the solution add a drop of 4 per cent cupric sulfate solution and shake. Add 6 ml. of concentrated sulfuric acid with agitation. Cool in ice and add 0.2 ml. of 1.5 per cent *p*-hydroxybiphenyl in 0.5 per cent sodium hydroxide solution. Incubate at 37° for 0.5 hour and heat in boiling water for 1.5

minutes to remove excess solid reagent. Cool to room temperature and read at 540 mμ against a water blank.

Alternatively use 0.002-0.01 mg. of serine and threonine in the sample and use a tube containing 1 drop of 4 per cent cupric sulfate solution and 6 ml. of concentrated sulfuric acid in place of the two sodium bisulfite tubes in the absorption train. Cool this absorption tube and add 0.2 ml. of the *p*-hydroxybiphenyl reagent before putting in place. Oxidize and aerate as previously described. Heat the tube in boiling water for 1.5 minutes and read at 540 mμ against a water blank.

Formaldehyde from serine. To the residue from the oxidation tube add 5 ml. of water. Prepare a sodium arsenite solution containing 0.495 gram of sublimed arsenious oxide, 4 ml. of 30 per cent sodium hydroxide solution, acidified to methyl red with 1:1 hydrochloric acid, and to which is added 6 grams of sodium bicarbonate. Add 10 ml. of this to reduce excess periodic acid and a few drops of bromocresol green indicator solution. Add 1:3 sulfuric acid until yellow, about 6 drops. Add the arsenite solution until green and about pH 4.8, and 1 ml. of excess arsenite solution. If too alkaline the formaldehyde will not distil, if too acid free iodine is liberated, distils, and interferes. Distil until nearly 40 ml. has gone over into 10 ml. of 0.2 per cent sodium bisulfite solution. Dilute tc 50 ml.

To a 5-ml. aliquot add 4 ml. of concentrated sulfuric acid with shaking and cooling in an ice bath. Add 1 ml. of fresh 5 per cent aqueous chromotropic acid solution and heat in boiling water for 30 minutes. Cool to room temperature and read at 540 mμ against a water blank.

2-Phenyl-1-aminopropionic Acid, Phenylalanine

Nitration of phenylalanine followed by reduction with hydroxylamine and ammonia results in the formation of a violet color.[37] Tyrosine, tyrosylglycine, glutamic acid, arginine, histidine, and protein hydrolyzates do not interfere in determination on blood and spinal fluids. Under conditions where histidine does interfere, it can be removed by sorption

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[37] Regine Kapeller Adler, *Biochem. Z.* **252**, 185-200 (1932); Richard J. Block and Diana Bolling, *J. Biol. Chem.* **129**, 1-12 (1939); George A. Jervis, Richard J. Block, Diana Bolling and Edna Kanze, *Ibid.* **134**, 105-13 (1940); Richard J. Block, George A. Jervis, Diana Bolling, and Merrill Webb, *Ibid.* **134**, 567-72 (1940); C. A. Knight and W. M. Stanley, *Ibid.* **141**, 39-49 (1941); W. L. Brown, *Ibid.* **155**, 277-82 (1944); Anthony A. Albanese and Dorothy L. Wagner, *Ibid.* **155**, 291-8 (1944); Jean Roche and Raymond Michel, *Bull. soc. chim. biol.* **28**, 844-7 (1946).

on Permutit. Trytophan interferes and has been determined by this method. It is best removed by precipitation with mercuric sulfate before the nitration step. It cannot be destroyed by acid hydrolysis as some of the phenylalanine is destroyed simultaneously.

An alternative[38] is to nitrate, reduce to the diamine, and react with 1,2-naphthoquinone-4-sulfonic acid to form a red compound. The product is insoluble in water but soluble in ethanol. Tryptophan is removed by treatment with potassium permanganate. Tyrosine, histidine, cystine, methionine, leucine, isoleucine, arginine, glutamic acid, alanine, glycine, proline, and valine do not interfere. Another satisfactory coupling agent is 1-naphthylamine acetate.[39] Tyrosine and tryptophan then interfere slightly.

Samples—*Blood and spinal fluid.* Precipitate proteins with 5 volumes of 10 per cent trichloroacetic acid solution, centrifuge, and wash the precipitate twice with 1 volume of 5 per cent trichloroacetic acid. Dilute the combined filtrate and washings to a known volume and use an aliquot as sample.

Proteins when tryptophan is present. Reflux 1 gram of sample in an oil bath at 125° with 16 ml. of 20 per cent sodium hydroxide for 24 hours. Add 24 ml. of 1:4 sulfuric acid slowly with stirring. Cool, dilute to 100 ml. with water, and add 400 mg. of kaolin. Shake well, centrifuge, and filter.

To an aliquot containing 1.5-2 mg. of phenylalanine diluted to 20 ml., add 6 ml. of 15 per cent mercuric sulfate solution in 1:4 sulfuric acid and place in a water bath for 10 minutes. Cool and add 4 ml. of 1:4 sulfuric acid. Dilute to 40 ml. with water, add 20 mg. of Celite, mix, and centrifuge for 5 minutes. Decant the supernatant liquid from the tryptophan-mercury precipitate. Precipitate the mercury from the liquid with hydrogen sulfide, centrifuge, and filter. To the precipitate add 12 ml. of water and a drop of 1:4 sulfuric acid and bubble through hydrogen sulfide again. Centrifuge and decant through the same filter.

To remove excess sulfuric acid, add about 8 grams of fresh barium hydroxide octahydrate, dissolved in 10 ml. of hot water. The solution should be acid to Congo red paper. Centrifuge and decant the supernatant liquid through a filter. Wash the barium sulfate residue with

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38 W. C. Hess and M. K. Sullivan, *Arch. Biochem.* **5**, 165-73 (1944).

39 Jean P. Zalta and Yvonne Khonvine, *Bull. soc. chim. biol.* **34**, 937-45 (1952).

50 ml. of hot water containing a drop of 1:4 sulfuric acid and a drop of capryl alcohol. Centrifuge and decant the supernatant liquid. Repeat the washing and centrifuging with 40 ml. of hot water and a drop of 1:4 sulfuric acid. Dilute to a known volume and use an aliquot.

Procedure—Evaporate a suitable aliquot of sample solution to dryness in a porcelain dish on a steam bath. Prepare a nitration mixture by dissolving 20 grams of potassium nitrate in 100 ml. of concentrated sulfuric acid.

Add 4 ml. of nitrating mixture to the residue and warm on a steam bath for 20 minutes. Add about 5 ml. of water and cool to 0°. Add 5 ml. of 30 per cent hydroxylamine hydrochloride. Cool again for 1 minute and dilute to 50 ml. with ice-cold concentrated ammonium hydroxide, running it down the side of the tube very slowly. Allow color to develop at room temperature for 45 minutes and read at 560 mμ against a reagent blank. If a precipitate forms, filter before making the reading.

2-(*p*-Hydroxyphenyl)-1-aminopropionic Acid, Tyrosine

The relatively stable aqueous solution of diazotized sulfanilic acid, *p*-phenyldiazonium sulfonate, gives a reproducible color with tyrosine, tyramine, imidazoles, cresols, and *p*-hydroxylphenyl acetic acid.[40] Therefore, for application of the method interfering substances, particularly tryptophan, must be separated. In neutral solution, tyrosine or tyramine give a nonspecific pink color with the reagent which changes sharply to yellow after about 30 seconds and fades. The yellow is not proportional to the concentration of tyrosine or tyramine. Addition of sodium hydroxide and later of hydroxylamine hydrochloride results in an intense bluish red color proportional to the amount of tyrosine or tyramine present.

Sodium or potassium chloride, sulfate, phosphate, acetate or citrate do not interfere up to 5 per cent. More than 0.5 per cent of ammonium salts or amino acids makes the color too yellow. Leucine must not exceed 10:1 or glycine 5:1 to the tyrosine or tyramine. The method is not directly applicable to a phosphotungstate filtrate because of other amino acids present, but tyrosine and tyramine are separated from such solutions and from each other. More than 5 mg. of tyrosine or tyramine is precipitated quantitatively by mercuric acetate with such

[40] Milton T. Hanke and Karl K. Koessler, *J. Biol. Chem.* 50, 235 ss (1922

small precipitation of other amino acids that they do not interfere. Sodium chloride and sodium sulfate do not interfere with the precipitation. The same technic gives another fraction for estimation of histidine and histamine.

Interfering substances which are removable include hydrogen peroxide, formaldehyde, acetaldehyde, acetone, acetoacetic acid, and ethyl, methyl and amyl alcohols. Not more than 1 per cent of glucose may be present.

Phosphotungstic-phosphomolybdic acid is quite generally known as Folin's phenol reagent.[41] A blue color is developed with this reagent by tyrosine but not by arginine, lysine, aspartic acid, alanine, phenylalanine, histidine, glutamic acid, proline, leucine, valine, glycine, asparagine, cystine, isoleucine, or isoserine. Indol gives a weak reaction. Tryptophan reacts like tyrosine but more slowly. This reagent cannot be used with 3,4-dihydroxyphenylalanine present as it gives the same reaction. Results are accurate to about 2 per cent.

The red color produced with mercuric nitrite and sulfuric acid, a modified Millon's reagent, is believed to be specific for the hydroxyphenol group.[42] The compound resembles that from Nessler's reagent and ammonia in many of its properties. The intensity and shade are governed to a large extent by the concentration of mercuric sulfate and sulfuric acid present. As the acidity is reduced, or mercuric sulfate increased, the intensity of color increases and the color becomes more violet. Precautions must therefore be taken to see that the same reagent concentrations are present in the sample as in preparation of the standard curve.

Removal of the tryptophan can be satisfactorily accomplished with mercuric sulfate if the acidity exceeds 3.5 per cent and is less than 7.5 per cent sulfuric acid. Chlorides interfere. By proper manipulation accuracy to 1.5 per cent is attainable. The presence of 3,4-dihydroxyphenylalanine will cause a faint yellow color upon addition of mercuric sulfate to the warmed sample. Upon cooling, this yellow compound precipitates and must be removed by centrifuging.[43]

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41 Otto Folin and W. Denis, *Ibid.* **12**, 245-51 (1912).

42 Otto Folin and Vintila Ciocalteu, *Ibid.* 73, 627-50 (1927); J. W. H. Lugg, *Biochem, J.* **31**, 1422-33 (1937); *Ibid.* **32**, 775-83 (1938); Erwin Brand and Beatrice Kassel, *J. Biol. Chem.* **131**, 489-502 (1939); D. M. Doty, *Ind Eng. Chem., Anal. Ed.* **13**, 169-72 (1941); E. Rauterberg, *Die Chemie* **56**, 91-2 (1943); *Z. ver. deut. Chem.* **48**, 121-2 (1944).

43 L. Earle Arnow, *J. Biol. Chem.* **118**, 531-7 (1937).

Tyrosine in sodium carbonate solution reacts with α-nitro-β-naphthol to yield a dark purple color.[44] As usually applied, the solution is made acid and the color reagent added in ethanol. With nitric acid present this gives a red coloration.[45] If tryptophan is present in greater amounts than tyrosine, it hinders the color development. Tryptophan can be destroyed during acid hydrolysis of proteins.

Sample—*Protein.* Hydrolyze a 3-gram sample of vacuum-dried protein by refluxing for 24 hours with 100 ml. of 1:6 sulfuric acid. Cool, dilute to about 1500 ml., and heat on a water bath. Add a hot solution of 89 grams of barium hydroxide in 500 ml. of water. Mix well and add 1:5 sulfuric acid, followed by 1 per cent barium hydroxide solution if necessary until only a slight excess of sulfate ion is present. Digest on the water bath for several hours, filter on a Büchner funnel while hot, and wash the precipitated barium sulfate with hot water. Concentrate on a steam bath until the residue is semi-solid.

Take up with 50 ml. of water and add 600 ml. of an 0.8 per cent solution of silver sulfate. Mix well and add a warm solution of 30 grams of barium hydroxide in 100 ml. of water. Transfer the brown mixture at once to an ice bath and allow it to settle for a half-hour. Under these conditions there is no appreciable reduction to metallic silver. Centrifuge the cold mixture and decant the clear colorless supernatant liquid from the brown precipitate. Acidify at once with 1:5 sulfuric acid to prevent reduction of the small amount of silver ion to free silver. Wash the solid residue from the silver precipitation with 50 ml. of a cold saturated solution of barium hydroxide. Acidify with 1:5 sulfuric acid and add the washings to the main bulk of the silver filtrate. This filtrate contains all of the tyrosine. Reserve the silver precipitate for recovery of histidine (page 138) and proceed with the solution.

Add sufficient excess of 1:5 sulfuric acid to the solution so that the precipitate of barium sulfate will settle readily. Add 1:10 hydrochloric acid drop by drop until silver chloride is no longer precipitated and a trace of excess chloride is present. Heat on a water bath and adjust with solutions of barium hydroxide and sulfuric acid until only a slight excess of sulfate ion is present. Digest for several hours, filter,

[44] Otto Gerngross, Karl Voss, and Hans Herfeld, *Ber.* 66B, 435-42 (1933).

[45] José Giral, *Anales inst. invest. cient. Univ. Nuevo León* 1, 113-20 (1944); Lloyd E. Thomas, *Arch. Biochem.* 5, 175-80 (1944); *Ciencia (Mex.)* 9, 127-30 (1948).

and wash. Concentrate the filtrate and washings on a steam bath to a semisolid mass which usually contains crystals of tyrosine.

Take up the semisolid residue in 75 ml. of water and add 1 ml. of glacial acetic acid and 3.5 grams of mercuric acetate. Reflux for 10 minutes or heat on a steam bath for 1 hour. A small amount of precipitate settles. Cool and add 7.5 grams of sodium chloride. Place in a refrigerator for 2 hours. Centrifuge with 20 ml. of a 10 per cent solution of sodium chloride for 5 minutes to separate tyrosinomercuric chloride, an insoluble white precipitate. Discard the clear supernatant liquid. Wash the solid residue with 0.25 ml. of 10 per cent sodium chloride solution. Transfer the washed solid quantitatively with 25 ml. of hot 1:4 hydrochloric acid to the flask in which it was precipitated with mercuric acetate. Heat on a water bath for 30 minutes. Usually the solid will completely dissolve. Add 50 ml. of water and saturate with hydrogen sulfide. Heat on a water bath for 30 minutes, filter on a small folded filter, and wash. Evaporate the filtrate and washings to dryness on a steam bath. Dissolve the pale brown crystals in water, dilute to 500 ml., and use an aliquot for development by *p*-phenyldiazonium sulfonate or phosphotungstic-phosphomolybdic acid.

Alternatively, gently reflux 1 gram of dried protein, 2 ml. of butanol to prevent foaming, silver wire or silver foil to prevent bumping, and 20 ml. of a 20 per cent solution of sodium hydroxide for 20 hours on a sand bath. Remove the condenser, add 10 ml. of water, and continue boiling for 10 minutes to remove the butanol. Withdraw the flame and add immediately, drop by drop, 10 ml. of 2:3 sulfuric acid. The addition of acid should produce boiling. This procedure is to prevent silicic acid from remaining in a colloidal form. Shake and cool. Add 5 ml. more of the 2:3 sulfuric acid and dilute to 100 ml. Filter and keep the filtrate on ice or in the dark. The sample can also be hydrolyzed on a micro scale.

To separate tryptophan place an 8-ml. sample of protein hydrolyzate in a small centrifuge tube. Add drop by drop, 4 ml. of 15 per cent solution of mercuric sulfate in 1:5 sulfuric acid. Let stand for 3 hours and then centrifuge for 5 minutes. Decant into a 100 ml. volumetric flask, drain, and rinse the edge of the tube with 2 ml. of 1:360 sulfuric acid. To the residue in the tube add 10 ml. of a 1.5 per cent solution of mercuric sulfate in 1:18 sulfuric acid. Stir and let stand for 10 minutes. Traces of tyrosine precipitated with the tryptophan are dissolved by this acid. Rinse off the stirring rod with 2 ml. of the same acid, centrifuge, and add the wash liquid to the original mother liquor.

Save the precipitated residue of tryptophan for determination by a separate procedure (page 134). Use an aliquot for color development by mercuric sulfate and nitrite.

Corn. Alkaline hydrolysis must be used since tryptophan is partially destroyed by acid hydrolysis in the presence of carbohydrate.

Prepare as for histidine (page 140) to "Filter the ammonia-free contents of the distilling flask and wash with hot water until free from chlorides." To the residue add 20 ml. of 20 per cent sodium hydroxide solution. Digest on a steam bath for 24 hours with a long air condenser. Add 10 ml. of 1:1 sulfuric acid and mix. Cool and dilute to 50 ml. Filter or centrifuge and treat with a few grams of acid-and-alkali-washed activated carbon. Filter and use aliquots for tyrosine and tryptophan by development with mercuric sulfate and nitrite.

Beer and wort.[46] De-gas if necessary and use. Simple peptides may introduce some error.

Procedure—*By p-phenyldiazonium sulfonate.* As reagent dissolve 4.5 grams of sulfanilic acid in 450 ml. of 1:9 hydrochloric acid by repeated shaking and dilute to 500 ml. with water. Mix 1.5 ml. of the sulfanilic acid solution with 1.5 ml. of 5 per cent sodium nitrite solution and immerse in an ice bath for 5 minutes. Add 6 ml. more of the 5 per cent sodium nitrite solution. Mix and let stand in the ice bath for 5 minutes. Dilute to 50 ml. and keep in an ice bath. Do not use for 15 minutes after diluting. The reagent must be prepared fresh daily.

To two 5-ml. portions of 1.1 per cent sodium carbonate solution, add 2 ml. of the reagent with a pipet which will deliver it in about 5 seconds. Mix by inclining the tubes. Exactly 1 minute later add 1 ml. of sample to one solution and 1 ml. of the appropriate standard solution to the other. Mix by inclining the tubes. Let stand exactly 5.5 minutes from the addition of sample and standard. Add 2 ml. of a 12 per cent solution of sodium hydroxide and mix. Exactly 1 minute later add 0.1 ml. of a 20 per cent solution of hydroxylamine hydrochloride and mix thoroughly. An intense bluish red color develops in 5-10 seconds, reaches its maximum in 30 seconds, and does not change for 30 minutes. Read within that period.

By phosphotungstic-phosphomolybdic acid reagent. Dilute an appro-

[46] H. Lüers, F. Stricker, and E. Schild, *Wochschr. Brau.* **55**, 33-6, 41-5 (1938).

priate amount of sample according to the tyrosine content to 30 ml. Mix well and add 20 ml. of saturated sodium carbonate solution. Make sure the solution is alkaline and add 2 ml. of 5 per cent sodium cyanide solution. Add 2 ml. of reagent (Vol. III, page 116), mix, and dilute to 100 ml. Let stand for 30 minutes and read against a reagent blank.

By mercuric sulfate and nitrite. Dilute a sample containing 0.25-0.9 mg. of tyrosine to 5 ml. and add 1.5 ml. of 1:6 sulfuric acid. Add 5 ml. of 10 per cent mercuric sulfate in 1:9 sulfuric acid and mix. Heat for 10 minutes at 100° and cool. Add 5 ml. more of the mercuric sulfate solution and dilute nearly to 25 ml. Add 0.1 ml. of 7 per cent sodium nitrite solution, mix, and dilute to 25 ml. The solution will be turbid if tryptophan or 3,4-dihydroxyphenylalanine is present. In that event, let stand for 50 minutes; in any event let stand for 10 minutes. Centrifuge if necessary and read against a reagent blank.

By α-nitroso-β-naphthol. Dilute the sample containing about 5 mg. of tyrosine to 5 ml. with water and add 1 ml. of a solution containing 12 per cent of α-nitroso-β-naphthol in ethanol. Follow with 1 ml. of concentrated hydrochloric acid and 1 ml. of 1:5 nitric acid solution. Stir after each addition and then stir vigorously for 5 seconds. Place in boiling water and continue to heat for 25 seconds after the appearance of color in the upper part of the tube. Cool in a water bath at 20-30° for a minute and dilute to 15 ml. with ethanol. Read after 5 minutes at 530 mμ against a reagent blank.

Dihydroxyphenyl-1-Aminopropionic acid, Dihydroxyphenylalanine

The yellow color of 3,4-dihydroxyphenylalanine with nitrous acid changes to orange-red in the presence of excess sodium hydroxide.[47] Tyrosine does not interfere in this determination. Sodium molybdate prevents rapid decomposition of the nitrous acid and causes a 50 per cent increase in color intensity. The color is stable for about 1 hour.

The reaction is given strongly by compounds with two or three phenolic groups, except orcinol, such as phloroglycinol, pyrogallol, resorcinol, epinephrine, and catechol. Only the latter two give the same red as the test substance. There is little or no color with ephedrine, phenol, and tyrosine.

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[47] L. Earle Arnow, *J. Biol. Chem.* **118**, 531-7 (1937).

Procedure—Mix 1 ml. of unknown, containing 0.02–1 mg. of 3,4-dihydroxyphenylalanine with 1 ml. of 1:20 hydrochloric acid solution. Add 1 ml. of a reagent containing 1 per cent of sodium nitrite and 10 per cent of sodium molybdate. Convert the yellow color to red with 1 ml. of 4 per cent sodium hydroxide solution and dilute to 5 ml. Read at 510 mμ against a reagent blank.

2-Indolyl-1-aminopropionic Acid, Tryptophan

The types of reactions used for determination of tryptophan may be classified as (1) reactions with aldehydes in acid solution, (2) oxidation of the condensation product with glyoxylic acid, (3) reaction with a mercury salt and nitrous acid, and (4) coupling with a diazotized amine.

Tryptophan condenses with *p*-dimethylaminobenzaldehyde to give a colorless compound which can then be oxidized with nitrite to give a blue color. Where in some of the early work this developed by air oxidation it is most widely developed by adding nitrite.[48] The oxidation of the condensation product should go on in the dark because destruction of tryptophan in acid solution is accelerated by light. However, color development has been reported as accelerated by sunlight and inhibited by iron.[49] The maximum color is obtained with free tryptophan in 12-13.2 N sulfuric acid at 25°. When starting with the protein, the optimum is at 19 N. The color developed in 1 hour approaches that obtained in 24 or 48 hours. Chloride increases the intensity of color developed but necessitates additional nitrite ion. Sodium bisulfite may decrease the color by 10 per cent. Either is removed with silver sulfate.

[48] E. Rohde, *Z. physiol. Chem.* **44**, 161 (1905); Elizabeth Kurchin, *Biochem. Z.* **65**, 451-9 (1914); C. E. May and E. R. Rose, *J. Biol. Chem.* **54**, 213-6 (1922); M. X. Sullivan, H. S. Milone, and E. L. Everitt, *Ibid.* **125**, 471-4 (1938); D. M. Doty, *Ind. Eng. Chem., Anal. Ed.* 13, 169-72 (1941); Henry S. Milone and Edward L. Everitt, *Proc. Soc. Exptl. Biol. Med.* **51**, 82-3 (1942); Jacob R. Spies and E. J. Coulson, *J. Am. Chem. Soc.* **65**, 1720-5 (1943); M. X. Sullivan and W. C. Hess, *J. Biol. Chem.* **155**, 441-6 (1944); Jacob R. Spies and Dorris C. Chambers, *Anal. Chem.* **20**, 30-39 (1948); **21**, 1249-66 (1949); Edward Steers and M. G. Sevag, *Ibid.* **21**, 641-2 (1949); A. Lesuk, U. S. Pharmacopoeia Amino Acids Advisory Committee, "Report on Collaborative Study on Chemical Tests and Standards for Amino Acids," Letter 135 (November 26, 1948); Jacob R. Spies, *Anal. Chem.* **22**, 1447-9 (1950).

[49] Yataro Obata, Sadao Sakamura, and Noboru Kakizaka, *J. Agr. Chem. Soc. Japan* **26**, 202-5 (1952).

Under properly selected conditions, the color conforms to Beer's law. Indole does not interfere but skatole does to give 1.6 times the color of tryptophan. Tryptamine gives 2.6 times the color of tryptophan. The wave lengths of maximum color development are not the same. Hypaphorine gives a red color. Glucose does not interfere but fructose gives some color, the more the longer the time of development. Age of the sodium nitrite is of little significance, but for maximum accuracy the solution of *p*-dimethylaminobenzaldehyde should be fresh.

Hydrolysis of proteins has usually been with sodium hydroxide, barium hydroxide, or sodium stannite in sodium hydroxide solution.[50] Barium ion, tin, and dissolved glass must be removed. Added cysteine prevents oxidative destruction.[51] Drastic hydrolysis by autoclaving for 5 hours or 10 hours in 20 per cent sodium hydroxide[52] give low results unless conducted in a hydrogen atmosphere. Purified hydrogen gives but little higher values than the commercial grade.

Nitrate may replace nitrite for oxidation.[53] Addition of gelatine to the sample causes full color development over the period 10-90 minutes and 20 per cent greater color intensity than in its absence.[54]

Another aldehyde reaction, one which is probably more sensitive, is that with vanillin.[55] The technic is specific for distinguishing tryptophan from its decomposition products. Indol and skatol give colored compounds. Neither prolines nor less than 2 per cent of sodium ion affects the color. More than 0.3 per cent of chloride ion interferes. The presence of mercury intensifies the color. The same oxidation of the colorless compound can be produced by light instead of nitrite[56] but with somewhat less convenience.

As might be expected, other oxidizing agents can be used, such as

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[50] E. Herzfield, *Biochem. Z.* **56**, 259-66 (1913); Erwin Brand and Beatrice Kassell, *J. Biol. Chem.* **131**, 489-502 (1939); Raymond Michel and Jean Schiller, *Bull. soc. chim. biol.* **27**, 456-8 (1945).

[51] K. A. Kuinan, C. M. Lyman, and F. Hale, *J. Biol. Chem.* **171**, 551-64 (1947).

[52] M. Gunness, I. M. Dwyer, and J. L. Stokes, *Ibid.* **163**, 159-68 (1945).

[53] R. W. Bates, *J. Biol. Chem.* **119**, VII (1937).

[54] Claire E. Graham, Edward P. Smith, Stanley W. Hier, and David Klein, *Ibid.* **168**, 711-6 (1947).

[55] Ida Kraus, *Ibid.* **63**, 157-78 (1925); S. M. Strepkov and I. M. Mavlianov, *Bull. biol. méd. exptl.* URSS **6**, 242-5 (1938); Pekka Brummer, *Acta Soc. Med. Fennicae ''Duodecim''* **A22**, 13-29 (1940).

[56] Joseph R. Spies and Dorris C. Chambers, *Anal. Chem.* **22**, 1209-10 (1950).

hydrogen peroxide.[57] Other aldehydes used are formaldehyde,[58] benzaldehyde,[59] and salicylic aldehyde.

Tryptophan condenses with glyoxylic acid by a different mechanism than with other aldehydes.[60] Color is then developed on oxidation in strongly acid solution.[61] The reaction is not specific for tryptophan, since the stable color is given with other indole derivatives. The maximum coloration is reached with two moles of copper to one of tryptophan, but excess copper beyond that does no harm. The reaction is also dependent on the concentration of glyoxylic acid and reaches a maximum at molar proportions of glyoxylic acid and tryptophan. Sulfuric acid may be replaced by phosphoric acid. The reaction with copper added will determine 0.001 mg. per ml. The copper also lowers the effect of interfering substances. The absorption bands are shifted by the period of heating at 100°. At 540 mμ there is no shift by heating beyond 5 minutes, but the sensitivity is substantially greater at 570 mμ. Only hepaphorine interferes. Thus zein, proline, oxyproline, gelatine, acid-hydrolyzed casein, galactose, or casein phosphopeptone do not alter the reading.

Diazotizing of tryptophan with sodium nitrite and coupling with *N*-(1-naphthyl)ethylenediamine yield a red color.[62] Heat and exposure to sunlight must be avoided. The color can be extracted with organic solvent. Tyrosine gives an interfering yellow color if present in amounts greater than tryptophan. Phenylalanine, proline, histidine, riboflavin, and nicotinic acid do not interfere. Skatole and indole give a deep red

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[57] M. X. Sullivan, H. S. Milone, and E. L. Everitt, *J. Biol. Chem.* **125**, 471-4 (1938).

[58] M. E. Voisenet, *Bull. soc. chim.* **33**, (3), 1198 (1905); J. Tillmans and A. Alt, *Biochem. Z.* **164**, 135-62 (1925); *Ibid.* **178**, 243-4 (1926); Enrico Poli, *Diagnostica tec. lab.* (Napoli), *Riv. Mens.* **10**, 260-3 (1939); Hans Herrmann and Wilhelm Ruff, *Weim. Arch. inn. Med.* **34**, 41-56 (1940); Th. Ruemele, *Z. Untersuch. Lebensm.* **79**, 453-8 (1940); A. Rossi and A. Vescia, *Boll. soc. ital. biol. sper.* **17**, 129-30 (1942).

[59] C. Reichl, *Monat.* **10**, 317 (1889); *Ibid.* **11**, 155 (1890); W. R. Fearon, *Biochem. J.* **14**, 548-64 (1920).

[60] J. L. D. Shaw and W. D. MacFarlane, *J. Biol. Chem.* **132**, 387-92 (1940).

[61] A. Adamkiewicz, *Arch. Physiol.* (Pflügers) **9**, 156 (1874); F. G. Hopkins and S. W. Cole, *Proc. Roy. Soc.* (London) **68**, 21 (1901); J. L. D. Shaw and W. D. McFarlane, *Can. J. Res.* **B16**, 361-8 (1938); D. C. Carpenter, *Anal. Chem.*, **20**, 536-8 (1948); M. Staub and H. Bosshardt, *Mitt. Gebiete Lebensm. Hyg.* **42**, 458-62 (1951).

[62] R. R. Nichols and H. W. Eckert, *N. Y. State Dept. Health, Ann. Rept., Div. Lab. and Research* **1941**, 26-7; H. William Eckert, *J. Biol. Chem.* **148**, 205-12 (1943).

color, phenol and cresol give a yellow to orange-red color, and *o*-, *m*-, and *p*-aminobenzoic acid give a deep red color. Dimethyl-α-naphthylamine as 0.4 per cent ethanolic solution may be substituted as reagent but requires longer for color development. A similar reaction is given by N-(1-naphthyl)diethylpropylenediamine.[63]

If tryptophan is diaminized by dilute acetic acid and sodium nitrite and then treated with thymol, a deep red color is obtained which is proportional to the quantity of tryptophan present.[64] Like tyrosine, tryptophan is estimated by the blue color given with phosphotungstic-phosphomolybdic acid, Folin's phenol reagent.[65] The proportionality is molar. Tryptophan must be isolated from hydrolyzates.

For determination by ferric chloride in the presence of trichloroacetic acid, follow the technic for indoleacetic acid (Vol. III, page 342). For development with mercuric sulfate and nitrite, follow the detail of the method for tyrosine (page 127). These methods are applicable only to isolated tryptophan fractions.[66]

One mole of tryphtophan reacts with four atoms of bromine and the resulting bromotryptophan is extractable into chloroform for reading the red color.[67] A lemon-yellow on adding mercuric acetate to the solution of tryptophan in 1:1 sulfuric acid is also readable.[68]

In the ultraviolet the absorption curves of tryptophane and tyrosine cross at 257.15 mμ and 294.4 mμ. By suitable selection of the wave length each can be determined in the presence of the other.[69]

Tryptophan and citrulline are the only amino acids giving a yellow color with diacetylmonoxime.[70] The color formation is complex. Other amino acids give pink. The color developed in mixed nitric and sulfuric acids is proportional to the typtophan and tyrosine content.[71]

When treated with 70-72 per cent perchloric acid at room tempera-

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63 Joseph Tabone, Colette Magis, and Jacqueline Troestler, *Bull. soc. chim. biol.* **29**, 1054-68 (1947).

64 Anthony A. Albanese and Jane E. Frankston, *J. Biol. Chem.* **144**, 563-4 (1942).

65 Otto Folin and Vintila Ciocalteu, *Ibid.* **73**, 627-50 (1927).

66 J. W. H. Lugg, *Biochem. J.* **31**, 1422-33 (1937); *Ibid.* **32**, 775-83 (1938).

67 Togo Simada, *J. Biochem.* (Japan) **33**, 17-34 (1941).

68 C. S. Runti, *Boll. soc. ital. sper.* **22**, 622-3 (1946).

69 T. W. Goodwin and R. A. Morton, *Biochem. J.* **40**, 628-32 (1946).

70 V. N. Orekhovich and A. A. Tustanovskiĭ, *Doklady Akad. Nank.* USSR **67**, 333-6 (1949).

71 J. Tillmans, P. Hirsch, and F. Stoppel, *Biochem. Z.* **198**, 379-401 (1928); G. Secchi, *Rend. ist. super. sanità* **5**, 932-43 (1942).

ture, tryptophan gives a yellowish-green fluorescence.[72] The sensitivity of the reaction is increased by a stabilizer. While not given by any other amino acid, some compounds related to tryptophan react. The color reaction with many compounds is altered by addition of ferric chloride. Casein, albumin, human blood serums, pepsin, crystalline soybean protein, gelatin, and silk do not give the test. Zein in 80 per cent ethanol gives a slight fluorescence indicating only a trace of tryptophan. Results on samples containing 0.006-0.008 mg. of tryptophan are accurate to ±5 per cent.

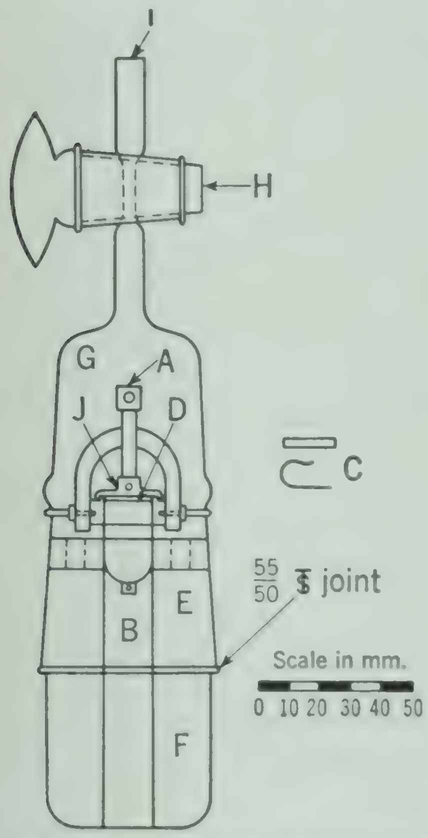

FIG. 8. Hydrogen filling apparatus

Sample—*Protein.* Transfer a sample expected to contain about 2-5 mg. of tryptophan to the nickel cup of a Parr 3-ml. microbomb. Add 1 ml. of 20 per cent carbonate-free sodium hydroxide. Work with a small gold spatula to remove occluded air and leave the spatula in the cup.

Place the cup in the bomb assembly as shown in Figure 8. Place the cap J in position with a new gasket and tighten the screw A to seat the gasket. Loosen A and place in tube 8 of the hydrogen filling apparatus. Place clip C under the edge of the cap at D to permit evacuation of air and admit hydrogen. The apparatus is made from a 55/50 standard taper joint. E is a perforated cork sheet supporting tube B which in turn holds the bomb. Support F in a clamp and hold cover G in place on F with a two-pronged clamp fitting over H. Lubricate the joint with stopcock grease. Connect I through pinchcocks and 2-way stopcocks to an aspirator and at least a 1-liter gasometer containing hydrogen over distilled water.

Evacuate to 35-40 mm. and fill with hydrogen. Repeat 3 times to

[72] Henry Tauber, *J. Am. Chem. Soc.* 70, 2615 (1948); *J. Biol. Chem.* 177, [illegible] (1949); Malcolm Gordon and Herschel K. Mitchell, *Ibid.* 180, [illegible] (1949).

remove residual air. Do not evacuate below 35 ml. to avoid loss of solution by bubbling.

Fill the apparatus with hydrogen under about 250 mm. water pressure so that outflow of hydrogen prevents entry of air while sealing. Close H and remove C with a strong magnet. Seat cap J and tighten A with a 3-mm. steel rod. Place the bomb in a holder which keeps it upright. Heat in an oven for an appropriate period. At 100 ±2° the hydrolysis of conalbumin takes 18 hours; of ovalbumin or edestin, 42 hours. At 151 ± 3° casein, β-lactoglobulin, ovalbumin, and edestin require 18 hours.

After hydrolysis, cool to room temperature and open. Remove the cup, and from it the gold spatula. Rinse this and the cup, add 1 ml. of 20 per cent sodium hydroxide solution, and dilute to 10 ml. Filter through an inorganic filter and use an aliquot as sample for development with *p*-dimethylaminobenzaldehyde and nitrite. If the color is not normal, let the solution stand for 6 hours and develop another aliquot. Alternatively develop with vanillin or glyoxylic acid.

For deamination and development with thymol, reflux a sample containing about 5 mg. of tryptophan for 20 hours with 5 ml. of 20 per cent sodium hydroxide solution per gram of sample. Neutralize to pH 7 with glacial acetic acid, filter silica dissolved from the glass by alkali, and dilute to 10 ml.

Protein with gelatin added. Reflux for 2 hours a mixture of 35 mg. of gelatin, 2 ml. of 4 per cent sodium hydroxide solution, and protein containing 0.1-0.4 mg. of tryptophan. Cool to room temperature and develop with *p*-dimethylaminobenzaldehyde and nitrate.

Protein or bacteria. Digest a sample expected to contain 0.02-0.12 mg. of tryptophan with 2 ml. of 20 per cent sodium hydroxide solution for 1 hour at 56°. This is ready for use as sample for development by *p*-dimethylaminobenzaldehyde and nitrate.

Casein. Treat as for protein in the technic above but use 4 per cent sodium hydroxide solution. Develop with *p*-dimethylaminobenzaldehyde and nitrate. Alternatively dissolve in 20 per cent sodium hydroxide or 5 per cent trichloroacetic acid by heating for a few minutes and develop with glyoxylic acid.

Foods containing proteins.[73] Alkaline hydrolysis cannot be used in the preparation of protein foods containing carbohydrates because the

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[73] Millard J. Horn and D. Breeze Jones, *Ibid.* **157**, 153-60 (1945).

latter react to give dark and colored solutions. As enzyme extract shake 2 grams of commercial papain with 100 ml. of water for 2 minutes and filter.

Treat 1-3 grams of the material, depending on the nitrogen content, with 35 ml. of 0.2 per cent sodium hydroxide solution, 10 ml. of papain solution, and 10 drops of a 5 per cent sodium cyanide solution. Stopper and incubate at 70° overnight. Carbohydrates and crude fiber remain in suspension. Add a small amount of Celite, shake, and filter. Wash the filter with water and dilute the clear yellow filtrate to 100 ml. Use an aliquot for development with *p*-dimethylaminobenzaldehyde and nitrate.

Tryptophan solutions. Chloride or bisulfite present. Treat a 10-ml. sample containing 0.5-1 mg. of tryptophan with a gram of pulverized silver sulfate. Shake frequently for a half-hour at which time the precipitate should settle readily. Centrifuge and develop with *p*-dimethylaminobenzaldehyde and nitrite.

Tryptophan precipitates. Tryptophan was isolated from tyrosine by precipitation with mercury (page 126). Dissolve the precipitate in 2 ml. of 1:1 sulfuric acid for development with vanillin or glyoxylic acid. Dilute to a volume which will give an appropriate concentration and so modify the procedure as to allow for the acid in the sample.

For development with phosphotungstic-phosphomolybdic acid, dissolve the precipitate in 10 ml. of 1:10 hydrochloric acid. Heat in boiling water for 10 minutes and pass a slow current of hydrogen sulfide through the hot solution until precipitation of mercuric sulfide is complete. Rinse the stirring rod and delivery tube with 2 ml. of 1:1 sulfuric acid. Stopper the tube and set aside for an hour. Centrifuge and decant. Wash the edge of the tube with 2 ml. of 1:360 sulfuric acid. Add 10 ml. of 1:360 sulfuric acid to the residue and stir. Centrifuge again and add about 15 ml. of water. Boil for 5 minutes to remove hydrogen sulfide, cool, and dilute to 100 ml.

Beer and Wort. See under tyrosine (page 126).

Blood serum. Dilute 0.1 ml. of blood serum to 1 ml. for development with glyoxylic acid.

Scabs.[74] Extract a 0.5-gram dried and powdered sample with ether. Dry and hydrolyze with 20 ml. of 20 per cent potassium hydroxide at

[74] Bernhard Zorn, *Z. physiol. Chem.* **285**, 143-5 (1950).

40°. Centrifuge and decant. Redisperse the precipitate, centrifuge, and decant. Dilute the centrifuge to 30 ml. for development with glyoxylic acid.

Procedure—*By p-dimethylaminobenzaldehyde and nitrite.* Prepare 23.8 *N* sulfuric acid with analytical accuracy. Add 1 ml. of 1:18 sulfuric acid containing 0.03 gram of *p*-dimethylaminobenzaldehyde to 8 ml. of this acid. Mix with 1 ml. of sample, cool, and store in the dark at 25° for 4 hours. Add 0.1 ml. of 0.07 per cent sodium nitrite solution, shake, and let stand in the dark for 30 minutes. Read at 600 mμ against a reagent blank. If there is any doubt of the presence of sufficient nitrite, add 0.1 ml. more of the 0.07 per cent solution and read again after 15 minutes. There should be no decrease in transmittance. Too much nitrite alters the color.

By p-dimethylaminobenzaldehyde and nitrate. Mix 1 ml. of alkaline digestate containing 0.006-0.06 mg. of tryptophan with 0.1 ml. of 5 per cent solution of *p*-dimethylaminobenzaldehyde in 1:9 sulfuric acid. Add 0.04 ml. of 2 per cent sodium nitrate solution and 5 ml. of concentrated hydrochloric acid. Heat at 56° for 1 minute and let stand for 15 minutes. Dilute to 10 ml. with 1:1 hydrochloric acid and again let stand for 15 minutes at room temperature. Dilute to 10 ml. with 1:1 hydrochloric acid solution and allow to stand for an additional 15 minutes. If the salt which forms upon addition of the acid to the alkaline solution does not dissolve after final dilution, filter the solution. Read at 560 mμ against a reagent blank.

By vanillin. Mix 1 ml. of sample containing 0.1-0.3 mg, of tryptophan with 0.1 ml. of 0.02 per cent sodium sulfide solution. Add 0.1 ml. of 0.1 per cent vanillin solution and chill. Add 3 ml. of concentrated sulfuric acid and place in a boiling water bath for 4 minutes. Let the solution stand for 4 hours and read at 530 mμ against water.

By glyoxylic acid. Mix an aqueous sample containing 0.02-0.15 mg. of tryptophan, 0.2 ml. of 0.4 per cent glyoxylic acid solution, and 0.3 ml. of 0.25 per cent copper sulfate pentahydrate solution. Dilute to 3 ml., place in an ice bath, and add 5 ml. of concentrated sulfuric acid dropwise with constant shaking. When cooled to room temperature, let stand for 20-30 minutes and immerse in boiling water for 20 minutes. Cool, dilute to 10 ml. with 3:5 sulfuric acid, and mix thoroughly. Read at 570 mμ against a reagent blank.

By N-(1-naphthyl)ethylenediamine. Dilute a sample containing 0.01-0.25 mg. of tryptophan to 4.5 ml. and add 0.5 ml. of concentrated hydrochloric acid, allowing for any already present. Add 1 ml. of 1 per cent aqueous sodium nitrite and swirl occasionally. After 30 minutes add 4 ml. of 4 per cent ammonium sulfamate solution and mix. After 10 minutes, add 10 ml. of water and 5 ml. of 0.1 per cent N-(1-naphthyl)-ethylenediamine dihydrochloride solution. Read the color after exactly 30 minutes. As blank use a sample in which the final reagent was replaced with water, or a developed sample decolorized with sulfite or nitrite. If the sample is highly colored, add 5 grams of sodium chloride and extract the developed color with 10 ml. of *n*-butanol.

By thymol. Mix a 2-ml. sample containing about 1 mg. of tryptophan with 0.3 ml. of 1 per cent aqueous sodium nitrite and 0.1 ml. of 10 per cent acetic acid solution. Shake intermittently for 10 minutes. Then add successively, with thorough mixing after each additon, 0.3 ml. of 1 per cent aqueous potassium persulfate, 0.5 ml. of 1 per cent thymol in ethanol, and 5 ml. of a mixture of 3 parts of 40 per cent trichloroacetic acid and 2 parts of concentrated hydrochloric acid. Heat in boiling water for 5 minutes and chill in an ice bath for 5 minutes. The sample will separate with 2 layers—a colorless layer above and a red layer of ethyl trichloroacetate below. Remove the upper layer as completely as possible and dilute the lower layer to 5 ml. with glacial acetic acid. Read at 540 mμ against a reagent blank.

By phosphotungstic-phosphomolybdic acid. Dilute a sample containing about 1 mg. of tryptophan to about 25 ml. and add 25 ml. of a saturated solution of sodium carbonate. Dilute to about 70 ml. and add 5 ml. of reagent (Vol. III, page 116). Let stand for 30 minutes and dilute to 100 ml. Read against a reagent blank.

Fluorimetrically by perchloric acid. As a stabilizer of the fluorescence mix 1 ml. of 1.17 ppm. aqueous indole solution, 1 ml. of 0.1 ppm. aqueous γ-(indole-3)-*n*-butyric acid, and 1 ml. of 10 per cent acid-hydrolyzed casein. Dilute to 10 ml. with 14.7 per cent disodium phosphate solution. Adjust the sample to pH 7.5-8 with phosphate buffer (Vol. I, page 176). Mix 0.4 ml. with 0.1 ml. of the stabilizer and add 2 ml. of 70-72 per cent perchloric acid. Mix, incubate for 1 hour at 40°, and cool to room temperature. Read fluorimetrically against a reagent blank.

2-Imidazolyl-1-Aminopropionic Acid, β-Imidazolyl-α-Alanine, Histidine

The method for histamine with diazotized sulfanilic acid is also applicable to histidine.[75] The first color is yellow but changes to red in 2-3 minutes. Fading starts in 5-6 minutes. More reliable results are obtained if the alkaline salt is added after histidine and the reagent have been mixed.

The reaction is probably the most reliable of all colorimetric reactions for amino acids, considering interference by tyrosine, guanine, adenine, phenols, imidazoles, sulfides, and ammonium salts. The presence of glycine, aspartic acid, and uric acid tends to inhibit color development unless the amount of reagent is increased. Urea does not interfere. It is probable that histidine and diazobenzene sulfonic acid react even in silghtly acid solution, since the color intensity is increased by a period of standing before addition of alkali. The reaction is probably deamination of histidine in the presence of nitrous acid and also coupling of the diazo compound with the imidazole nucleus. The reaction takes a course nearer to a stoichiometric relationship in slightly acid solution.

The reagents selected do not give the maximum possible color intensity, but rather the most easily reproducible color intensity. The use of too much alkali destroys the color. This color is directly proportional to the histidine content when the sample contains 0.005-0.05 mg. per ml. For satisfactory results the sample must not have an acidity or alkalinity greater than 0.01 *N*. The presence of 0.01 mg. of mercuric chloride, or of copper and silver salts interferes. Moderate amounts of ferric chloride or sodium chloride do not. Small amounts of phosphotungstic acid do not interfere. This amino acid can be sorbed on an ion-exchange resin and eluted with 5 per cent acetic acid.[76]

Other diazo reagents are formed from *p*-nitrophenylantidiazotate;[77] *p*-monochloroaniline,[78] and *p*-nitroaniline.[79]

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[75] Moriz Weiss and Nikolaus Ssobolew, *Biochem. Z.* **58**, 119-29 (1913-14); K. K. Koessler and Milton T. Hanke, *J. Biol. Chem.* **39**, 521-38 (1919); Milton T. Hanke and K. K. Koessler, *Ibid.* **43**, 527-42 (1920); Milton T. Hanke, *Ibid.* **66**, 475-88 (1925); Herbert T. MacPherson, *Biochem. J.* **36**, 59-63 (1942).

[76] P. Decker, H. Wagner, H. Riffart, and H. Breher, *Klin. Wochschr.* **29**, 642 (1951).

[77] W. Diemair and H. Fox, *Biochem. Z.* **298**, 38-50 (1938).

[78] S. Edlbacher, H. Baur, H. R. Staehelin and A. Zeller, *Z. physiol. Chem.* **270**, 158-64 (1941).

[79] A. D. Marenzi and F. Villalonga, *Rev. soc. argentina biol.* **19**, 43-54 (1943).

A color produced by histidine and sulfanilic acid in sulfuric acid followed by neutralization, is suitable for colorimetric estimation.[80] When a histidine solution is warmed with 1 per cent bromine in 1:2 acetic acid, it gives a red color [81] which forms a black precipitate unless treated with ammonium hydroxide and ammonium carbonate. Then a blue-violet color develops.[82] The same color is given by histamine and other derivatives. The color, sensitive to 1 part in 50,000, is due to formation of a complex ring compound. The reaction is not given by imidazol-propionic, -acetic, or -lactic acid, carnosine, or methyl histidine. Histamine gives only a faint yellow color. Methyl histidine gives a color of about one-fifth the intensity of histidine. Tryptophan, tyrosine, uracil, proline, hydroxyproline, methionine, glycine, and alanine interfere. Histidine is desirably separated as the silver or mercury salt. Bromine can be replaced by potassium bromide and potassium bromate in acid solution.[83] Organic solvents such as pyridine and acetone do not then interfere.

In determinations on acid protein hydrolysates or in biological fluids, the color may be obscured by a brown tinge. One way of overcoming this is to oxidize with 0.3 per cent potassium permanganate in sulfuric acid before treatment to develop color. Histamine gives a weak golden yellow color and methylhistidine a weak red-violet. There is no interference from imidazoles of propionic, lactic, or acetic acids, or from carnosine. The reaction is sensitive to 1:50,000.

In a modified form of this reaction copper is added and modifies the color.[84] In that version, amino acids slightly enhance the color of amounts as small as 0.08 mg. of histidine but, with larger amounts such as 0.32 mg., have the opposite effect. Creatinine also interferes in much the same way, but creatine is without effect. Urea greatly reduces color development. Tryptophan and tyrosine give obscuring colors of their own. Cystine, methionine, and zinc each greatly reduces

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[80] Kozo Suzuki and Yoshio Kaisho, *Bull. Agr. Chem. Soc. Japan* **3**, 33-4 (1927).

[81] Franz Knoop, *Beitr. Chem. Physiol. Path. Hofmeister* **11**, 356 (1908).

[82] Regine Kapeller-Adler, *Biochem. Z.* **264**, 131-41 (1933); *Ibid.* **571**, 206-8 (1934); D. W. Wooley and W. H. Peterson, *J. Biol. Chem.* **122**, 207-11 (1937); Fr. Niendorf, *Z. physiol. Chem.* **259**, 194-200 (1939); E. Racker, *Biochem. J.* **34**, 89-96 (1940); D. M. Doty, *Ind. Eng. Chem., Anal. Ed.* **13**, 169-72 (1941); Eugenio Bonetti, *Boll. soc. ital. biol. sper.* **20**, 46-8 (1945); Cf. Zenya Kurihara, *J. Biochem. (Japan)* **30**, 205-16 (1919); Cf. K. Schmid, *Helv. Chim. Acta* **29**, 226-8 (1946).

[83] D. A. Tsuverkalov and M. A. Torban, *Biokhimaya* **16**, 74-80 (1951).

[84] A. Hunter, *J. Biol. Chem.* **196**, 589-98 (1952).

the intensity of color development. The method cannot be applied to urine or to unfractionated protein hydrolyzates because of the presence of interfering substances. For neutral unbuffered histidine solutions, the method is accurate to about ±3 per cent.

Samples—*Proteins.* The treatment of vacuum-dried protein for isolation of histidine as a silver precipitate has been described (page 124). Mix the silver precipitate with 60 ml. of 1:35 sulfuric acid and 150 ml. of water. Add 3 ml. of concentrated hydrochloric acid and mix well. Heat on a steam bath for 15-30 minutes to complete the precipitation of silver chloride. Filter through a folded filter and wash the precipitate until the washings are free from chlorides. Neutralize the acidity of the filtrate with 10 per cent sodium hydroxide solution and dilute to 500 ml. The solution so obtained is free from interfering substances. Develop with *p*-phenyldiazonium chloride or with sulfanilic acid and alkali.

For development with bromine and ammonium carbonate, hydrolyze a sample approximating 2-3 grams of dry protein with 10 ml. of 1:8 sulfuric acid for 20 hours. Let cool and dilute to about 100 ml. Add barium carbonate until precipitation of the sulfate ion is complete. Let the precipitate settle and decant through a filter. Wash the precipitate several times. Dilute the combined filtrates to 250 ml.

Take 100 ml. of the hydrolyzed protein and evaporate this to small volume. Transfer the residue with a little water to a flask and add ethanol until turbidity or a faint precipitate appears. Add ether equal to about one-third the volume of ethanol. Add an excess of a 10 per cent solution of mercuric sulfate in 1:20 sulfuric acid. Mix and let the solution stand for 24 hours to precipitate. Filter by suction and wash with ethanol and then with ether. Dry in the air. Dissolve the air-dried powder in 50 ml. of hot 1:5 hydrochloric acid. Filter and wash the insoluble matter on the filter with 10 ml. of water. Saturate the solution with hydrogen sulfide. When precipitation is complete, filter, and wash the residue on the filter with water.

Neutralize the filtrate to litmus with 10 per cent sodium hydroxide solution and evaporate to dryness on a water bath. Dissolve the residue in 20 ml. of 1:9 sulfuric acid for use as sample. It still contains interfering substances which will be removed in the procedure.

Urine. Take 150-300 ml. as sample. Add cold saturated barium hydroxide solution in excess to precipitate the phosphate. Filter and add an excess of 1:10 sulfuric acid to the filtrate to precipitate the

excess of barium. Filter and neutralize the filtrate with a few drops of 10 per cent sodium hydroxide solution. Evaporate to small volume and treat as for protein by bromine, starting at "Transfer the residue with a little water . . . ."

Corn. Grind the air-dried sample to 1 mm., dry *in vacuo* at 100° for 5 hours, and extract with anhydrous ether for 48 hours in a Soxhlet apparatus. Discard the ether extract and grind the dried fat-free residue to 100-mesh in a ball mill. Heat 5 grams of residue with 150 ml. of water for 30 minutes on a steam bath to gelatinize the starch, and cool. Add 2 ml. of saliva and a few ml. of toluene. Stopper and incubate for 72 hours at 38°, shaking occasionally. Heat on a steam bath to inactivate the enzyme and drive off the toluene. Filter, wash with hot water, and discard the filtrate.

Add 20 ml. of 1:1 hydrochloric acid to the residue and follow with a few drops of capryl alcohol. Reflux gently for 20 hours on a sand bath. A "cold finger" is an adequate condenser. Remove the condenser, boil off the capryl alcohol, and filter humin. Wash thoroughly and evaporate the filtrate and washings to a thick paste on a boiling water bath *in vacuo* below 60°. Add 50 ml. of water and evaporate again to a thick paste. Repeat twice more to remove as much hydrochloric acid as possible. Add 25 ml. of water to the paste and make alkaline to litmus with solid calcium hydroxide. Add 25 ml. of ethanol and distil off the ammonia *in vacuo*. By quantitative absorption in standard acid, the ammonia nitrogen is determinable at this point.

Filter the ammonia-free contents of the distilling flask and wash with hot water until free from chlorides. Acidify the filtrate and washings with 1:1 hydrochloric acid, concentrate to 10 ml., and dilute to 50 ml. with water. Decolorize with a few grams of acid-and-alkali-washed activated carbon and filter. Use an aliquot of this solution for determination of cystine and cysteine (Vol. III, page 482 *et seq.*).

Precipitate the basic amino acids from a 25-ml. aliquot with 1 per cent phosphotungstic acid. Wash the phosphotungstic acid precipitate with ice-cold solutions in ice-cold equipment.

Add a thick suspension of barium hydroxide to the washed precipitate until just alkaline to litmus. Centrifuge the barium phosphotungstate and decant. Wash the residual precipitate in hot water and centrifuge. Combine the filtrate and washings and add 1:5 sulfuric acid dropwise until it is in slight excess. Digest the barium sulfate to make it filterable and filter. Wash with hot water and concentrate the

filtrate and washings *in vacuo*. Dilute to volume as the solution for determination of arginine and histidine.

Procedure—*By diazotized sulfanilic acid.* Dilute an amount of sample containing 0.01-0.2 mg. of 10 ml. Add 1:9 hydrochloric acid until acid to litmus and 1 ml. of 1 per cent sulfanilic acid solution in 1:9 hydrochloric acid. Add 1 ml. of 5 per cent sodium nitrite solution and after 30 minutes 3 ml. of 0.2 per cent sodium carbonate in 75 per cent ethanol. Add 10 ml. of ethanol and dilute to 25 ml. with water. Read at 530 mμ against a reagent blank.

By sulfanilic acid and alkali. To 10 ml. of aqueous solution containing histidine, add 2 ml. of a 2 per cent solution of sulfanilic acid in 1:9 sulfuric acid. Add 2 ml. of concentrated sulfuric acid and mix well. Add 10 per cent sodium hydroxide solution until alkaline and read against a reagent blank.

By bromine and ammonium carbonate. The sample solution is in 1:9 sulfuric acid. To 2 ml. of solution, add 0.3 per cent potassium permanganate solution drop by drop until a faint pink color remains. Let stand until this color disappears. Histidine is not oxidized under these conditions, but remaining interfering substances are. If necessary, heat for a few minutes on a water bath to dissolve any brown precipitate. Cool and add a 1 per cent solution of bromine in 1:2 acetic acid drop by drop. When a pale yellow color is obtained which does not disappear in 10 minutes, sufficient bromine is present. A large excess will cause low results. Excess may also be checked with potassium iodide-starch paper. Allow to stand for 10 minutes and then remove excess bromine by bubbling air through the solution until the yellow color disappears. Add 2 ml. of a solution made up of 2 parts of concentrated ammonium hydroxide and 1 part of 10 per cent ammonium carbonate solution and immerse in boiling water for exactly 5 minutes. Next, immerse in an ice bath for 5 minutes. Allow to stand at room temperature for 20 minutes and dilute to 10 ml. with ethanol. Mix and read within 15 minutes against a reagent blank.

By bromine and copper. Prepare a bromination reagent by dissolving 0.5 ml. of bromine in 50 ml. of tertiary butyl alcohol and dilute with water to 100 ml. This will keep in a refrigerator for 2-3 weeks. To 5 ml. of sample solution containing not more than 0.33 mg. of histidine add 0.5 ml. of 1:18 sulfuric acid, bring to 25°, and add, in a dim light, 0.25 ml. of bromine reagent. Stopper the tube containing the bromine-treated sample and place in the dark at 25° for 2.5 hours.

Destroy excess bromine by addition of 0.2 ml. of 3 per cent arsenous oxide in 1 per cent sodium hydroxide solution.

As a copper reagent add 2.1 ml. of glacial acetic acid and 0.26 ml. of a 1 per cent copper sulfate solution to 90 ml. of water. Dilute this to 100 ml. and dissolve 100 grams of sodium acetate in it. This gives about 172 ml. of solution. Add 1.2 ml. with shaking to the sample solution.

Let the treated sample stand for 15 minutes for maximum color development, dilute to 10 ml., and read against a blank at 545 mμ. The color is stable for 30-35 minutess after addition of the acetate.

By diazotized p-monochloroaniline. As reagent, mix 2 volumes of a 0.25 per cent solution of *p*-monochloroaniline in 1:40 hydrochloric acid with 1 volume of 0.5 per cent aqueous solution of sodium nitrite. Mix 5 ml. of 0.53 per cent sodium carbonate solution with 1 ml. of sample and add 0.5 ml. of diazo reagent dropwise. Leave in ice water for 3 minutes. Extract with 10 ml. of butanol. Filter the extract and read at 450 mμ against a reagent blank.

N-(α)-β-Alanyl-histidine, Carnosine

Carnosine gives the same intense red color with *p*-phenyldiazonium sulfonate[85] as histidine. The color does not conform to Beer's law. Both carnosine and its methyl derivative, ansarine, are precipitated from a protein-free extract by mercuric acetate and alcohol. Guanine, xanthine, histidine, and tyrosine are the only known non-protein muscle constituents which might interfere, but of these guanine and xanthine are probably present only in traces in mammalian muscle and histidine has not been so demonstrated. Tyrosine is believed to be responsible for about 2 per cent of the diazo color in such muscle extract. Alternatively, use the blue color of the copper compound of carnosine for its estimation.[86]

Carnosine is hydrolyzed to histidine and determined by the bromine and ammonium carbonate method. If free histidine is present, this must be estimated. If anserine is present, another method must be used,[87] in which a suitable correction is applied.

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[85] W. M. Clifford, *Biochem. J.* 15, 400-6 (1921); John A. Zapp, Jr. and D. Wright Wilson, *J. Biol. Chem.* 126, 9-17 (1938); For more details see page 137.

[86] O. Fürth and T. Hryntschak, *Biochem. Z.* 64, 172-94 (1916).

[87] Regine Kapeller-Adler, *Ibid.* 264, 131-41 (1933); Regine Kapeller-Adler and Fritz Haas, *Ibid.* 269, 263-70 (1934).

Sample—*Muscle.* Dissect a fresh sample, kept under refrigeration for less than 24 hours, and then extract. Free the muscle from excess fat and connective tissue and mince in a grinder. Extract a 15 to 50-gram sample 3 times at pH 5-6 with 3, 3, and 2 parts of acidified water for 0.5 hour each time at 60-70°. Concentrate the combined extracts on a water bath below the boiling point before a fan, to a known volume representing about 1 gram of muscle per ml. A small coagulum forms during the concentration. Remove and wash with centrifugation. Adjust an aliquot to pH 5-6 and add 5 volumes of boiling ethanol. Heat to boiling on a water bath. Traces of protein and glycogen precipitate, alcohol-insoluble proteins coagulate, and there is less chance of nitrogenous extractives being sorbed on the coagulum. More ethanol than indicated will precipitate carnosine and anserine. Neutralize the ethanolic filtrate and washings to litmus with 10 per cent sodium hydroxide solution and add an excess of saturated mercuric acetate solution to effect a separation of carnosine and anserine from the other extractives. Centrifuge, wash, and suspend the mercury precipitate in hot 1:350 sulfuric acid. Decompose it with hydrogen sulfide. Remove the mercuric sulfide by centrifugation and wash it repeatedly with 1:350 sulfuric acid. Remove sulfate from the combined centrifugates with barium hydroxide, then remove the barium sulfate, and concentrate the combined centrifugates to an accurately known volume representing about 1 gram of muscle per ml. Determine carnosine on an aliquot of this solution by the *p*-phenyldiazonium chloride.

Alternatively, with lesser accuracy, grind a 15-gram sample of fresh tissue with 50 ml. of water. Digest at 60° for 30 minutes. A single extraction is sufficient. Filter and press the liquid from the pulp. To 25 ml. of filtrate add 2 ml. of 20 per cent metaphosphoric acid and mix well. Let stand for 24 hours and filter. Wash, neutralize the filtrate with 10 per cent sodium hydroxide solution, and dilute to 100 ml. for development with *p*-phenyldiazonium chloride.

For development with bromine, free 100-150 grams of muscle from fat and connective tissue. Grind finely and extract with water at 70°. Decant the extract through a filter and repeat the extraction twice. Mix the extract with acetic acid to a concentration of 10 per cent to precipitate protein. Filter, evaporate to a small volume, and dilute to a known volume.

At this point apply the bromine and ammonium carbonate reaction (page 141) qualitatively to see whether free histidine is present in the

sample. If it is present, add an excess of a 10 per cent solution of mercuric sulfate in 1:20 sulfuric acid to precipitate the histidine. Mix well and let stand for 24 hours to precipitate. Filter by suction and wash with ethanol, then with ether. Dry in the air. Proceed from that point as for preparation of sample for histidine (page 139), starting at "Dissolve the air-dried powder in 50 ml. of hot 1:5 hydrochloric acid."

Procedure—*By p-phenyldiazonium chloride.* As reagent, mix 0.4 gram of sulfanilic acid, 4 ml. of concentrated hydrochloric acid, and 40 ml. of distilled water. Shake at intervals until solution is complete. Cool below 10° and add twice the volume of a 0.5 per cent solution of sodium nitrite. To 10 ml. of 1.1 per cent sodium carbonate solution, add 4 ml. of reagent. Mix and let stand for one minute. Add 1 ml. of sample solution. Mix, let stand for 5 minutes, and read against a reagent blank.

As the copper compound. Reflux 100 ml. of the sample solution with a few mg. of pure copper hydroxide for one minute. Filter through dry paper in a hot water funnel. Cool and read the color. Return the sample to the flask, add a little more copper hydroxide, and again boil for one minute. Filter another sample and read. If the color is deeper than in the first sample removed, repeat the treatment with copper hydroxide until the maximum possible coloration is obtained.

By bromine and ammonium carbonate. Determine as for histidine (page 141). If free histidine was found, subtract that value from the final result to give the histidine equivalent to the carnosine present. Multiply this by 1.46 to convert the results to carnosine.

Methylcarnosine, Anserine

Anserine, when hydrolyzed, gives methyl histidine. This gives no reaction by the diazo method for histidine. It does react by the bromine and ammonium carbonate method, unless removed, to give one-fifth the intensity of color given by histidine. The difference between results by the two methods is therefore the basis of estimation of anserine.[88]

Sample—Prepare the hydrolyzate as described for estimation of histidine by bromine and ammonium carbonate (page 139), omitting the precipitation by mercuric sulfate.

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[88] Regine Kapeller-Adler, *Biochem. Z.* **264**, 131-41 (1933); Regine Kapeller-Adler and F. Haas, *Ibid.* **269**, 263-70 (1934).

Procedure—In one portion of the hydrolyzate determine histidine by the bromine and ammonium carbonate method (page 141). The color developed is that of histidine and methyl histidine. In another portion of the hydrolyzate, determine the histidine by diazotized sulfanilic acid.

Subtract the second value from the first and multiply by 5. This is the value for the methyl histidine and therefore equivalent to the anserine content. Multiply by 1.3 to convert to anserine.

2-Hydroxy-1-aminobutyric Acid, Threonine

If threonine is oxidized with enough lead tetraacetate, acetaldehyde is formed in an amount proportional to the threonine used.[89] Upon reaction with warm concentrated sulfuric acid and *p*-hydroxydiphenyl an intense red-violet color results. The presence of not over 10 mg. of *d*-arginine hydrochloride, *l*-aspartic acid, *l*-cystine, *l*-dihydroxyphenylalanine, *d*-glutamic acid, glycine, *l*-histidine hydrochloride, *dl*-isoleucine, *dl*-leucine, *d*-lysine dihydrochloride, *dl*-norleucine, *dl*-phenylalanine, *l*-proline, *l*-tyrosine, and *dl*-valine does not interfere. *l*-Hydroxyproline and *l*-tryptophan can decrease the yield of acetaldehyde but this is overcome by adding more lead tetraacetate. A large quantity of alanine will interfere but is corrected from a separate determination of that amino acid. No detectable amount of acetaldehyde results from oxidation of arabinose, ascorbic acid, fructose, galactose, glucose, glycerol, lactose, maltose, and mannose. The reaction is sensitive to 0.004 mg. of threonine.

Oxidation with periodate gives acetaldehyde quantitatively, but it has been estimated titrametrically[90] rather than colorimetrically. The simultaneous determination of threonine and serine is described under the latter (page 119).

Sample—*Protein.* Reflux 2–100 mg. of protein overnight with 1–5 ml. of 1:4 hydrochloric acid solution. Remove excess acid by concentration *in vacuo* and bound acid by warming with an excess of freshly prepared aqueous silver oxide. Remove the silver with hydrogen sulfide and concentrate the amino acid to dryness. Take up the residue in

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[89] Richard J. Block and Diana Bolling, *Proc. Soc. Exp. Biol. and Med.* **40**, 710 (1939); *J. Biol. Chem.* **130**, 365-74 (1939).

[90] Leo A. Shinn and Ben H. Nicolet, *Ibid.* **138**, 91-6 (1941).

acetic acid and use an aliquot containing 0.5-5 mg. of protein for analysis.

Procedure—Set up six successive tubes with inlet tubes leading to near the bottom. Tube 1 contains 20 ml. of concentrated sulfuric acid to dry and clean incoming air. Tube 2 serves as a trap for sulfuric acid spray from tube 1. Tube 3, fitted with an interchangeable ground glass joint, contains the reaction mixture. It is kept in a water bath at 30°. The reaction mixture is 25 ml. of glacial acetic acid, containing 0.02-1.5 mg. of threonine, and at least 1 gram of lead tetracetate. Tube 4 kept at 4° is a trap for condensing acetic acid vapors. Tube 5 contains a 2-inch column of sodium hydroxide pellets to remove traces of acids which might distil over with the air containing the acetaldehyde. Change the pellets every second run. Tube 6, of the same construction as tube 3, contains 10 ml. of concentrated sulfuric acid, 5 drops of water, and 100 mg. of *p*-hydroxydiphenyl. Keep this tube at 0° in ice water.

Carry out the reaction by passing air for 1 hour. Keep tube 6 at 0° until ready to remove the excess *p*-hydroxydiphenyl. Do this by placing the disconnected tube in boiling water for exactly 2 minutes, immediately cooling to 0°, and then allowing to come to room temperature. The color will vary from red to violet. Read at 560 mμ against a reagent blank.

3-Carboxy-1-aminobutyric Acid, Glutamic Acid

Glutamic acid reacts with ninhydrin to yield β-formylpropionic acid, the 2,4-dinitrophenylhydrazone of which gives a reddish-brown color in alkaline solution.[91] Aspartic acid interferes and requires chromatographic separation of glutamic acid with acid aluminum oxide.[92] Cystine and cysteine form acidic hydrazones after reaction with ninhydrin. However, the error encountered here is small. Glutamine in amounts up to 0.05 mg. per ml. causes almost no interference.

Procedure—*Aspartic acid present.* Stir 10 grams of aluminum oxide in about 50 ml. of 1:10 hydrochloric acid for 1 minute, decant

[91] Blanche A. Prescott and Heinrich Waelsch, *J. Biol. Chem.* **164**, 331-43 (1946).
[92] T. Wieland, *Ber.* **75**, 1001 (1942).

the acid, and wash the oxide with water until the washings are neutral to litmus. Store this "acid" aluminum oxide under water. Prepare a sorption column of tubing 2.3×80 mm. i.d. with an 11×4.5 mm. reservior at the top. Place about 6 cm. of the acid aluminum oxide over a glass wool plug. Fill the rest of the funnel with water and centrifuge to pack down the oxide.

Neutralize the sample solution containing no more than 0.025 mg. of glutamic acid per ml. to pH 7 by adding 1 per cent sodium hydroxide solution. Pass 2 ml. of the solution through the sorbant. When the surface of the fluid is about 1 mm. above the surface of the oxide, add 2 ml. of water. Discard both filtrate and wash water. Elute by passing two 2-ml. portions of 1:35 acetic acid through the column. Use this sample as though no interferences had originally been present.

Aspartic acid absent. Adjust the sample to approximate neutrality and add about 3 per cent by volume of glacial acetic acid.

Procedure—To the sample add 20 ± 0.5 mg. of ninhydrin and immerse in boiling water for exactly 10 minutes. Transfer to an ice bath for 2 minutes. Add the following reagents successively at 5-minute intervals: 0.4 ml. of 14 per cent guanidine carbonate, 1 ml. of 12 per cent lead acetate solution, and 0.5 ml. of 20 per cent sodium hydroxide solution. Dilute to 6 ml. with water. Stir well, centrifuge for 10 minutes, and decant the supernatant liquid.

Prechill 3 ml. of a 0.1 per cent solution of 2,4-dinitrophenylhydrazine in 1:10 hydrochloric acid and leave in an ice bath. Place a glass tube, with a 1-mm. tip in the tube, and add 5 ml. of the centrifugate. Mix by passing air through for 1 second. After exactly 10 minutes, add 10 ml. of capryl alcohol and agitate by a vigorous stream of air for 1 minute. Remove from the ice bath and remove the aqueous phase by suction. Clarify the capryl alcohol layer by centrifuging for 5 minutes. To a 9-ml. aliquot of the capryl alcohol, add 6 ml. of borate buffer which contains 2 per cent of sodium tetraborate and 0.53 per cent of sodium carbonate adjusted to pH 10 if necessary with sodium hydroxide solution. Agitate vigorously for 1 minute with a stream of air and centrifuge for 20 minutes. Mix 5 ml. of the borate extract with 2 ml. of absolute ethanol and immerse in a water bath for 5 minutes at 25°. Mix with 1 ml. of 20 per cent sodium hydroxide solution and read after exactly 2 minutes at 420 mμ against a reagent blank.

Pteroylglutamic Acid, Pteroyl-3-carboxy-1-aminobutyric Acid, Folic Acid

Folic acid is N-[4{[(2-amino-4-hydroxy-6-pteridyl)methyl]-amino} benzoyl]glutamic acid. It is reduced, coupled, and read.[93] Chemically, folic acid is reduced in acid solution to *p*-aminobenzoyl glutamic acid. Such reduction with titanous chloride or with zinc[94] may go too far. Therefore, zinc amalgam is preferable.[95] The resulting amine in the 10-position is then coupled with dimethyl-*α*-naphthylamine or N-(1-naphthyl)ethylenediamine dihydrochloride. Early work used *p*-aminobenzoic acid as standard, but *p*-aminobenzoylglutamic acid, the actual product of reduction, is preferable. The color does not fully conform to Beer's law. Adenine, adenosine triphosphate, and nucleic acids interfere. Results are accurate to ±0.8 per cent.

Potassium permanganate oxidation of pteroylglutamic acid yields a strongly fluorescent substance, 2-amino-4-hydroxypteridine-6-carboxylic acid.[96] The increment of increased intensity of the fluorescence, on oxidation, is directly proportional to concentration, if fluorescent pigments are absent. Riboflavin and many of the pterins interfere. When such interefering substances are present, the oxidation product must be isolated chromatographically and its fluorescence determined directly. The method is accurate within ±6 per cent.[97] The fluorescence of the oxidation product does not vary for at least 10 days. Buffering is essential as the fluorescence is altered by pH changes outside the range 4-9.5. Salts alter the fluorescence.

Turbidimetric estimation of effect on the rate of growth of *Lacto-*

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[93] *United States Pharmacopoeia* XIV, pp. 250-1, Mack Printing Company (1950).

[94] B. L. Hutchings, E. L. R. Stokstad, J. H. Boothe, J. H. Mowat, C. W. Waller, R. B. Angier, J. Semb, and Y. Subbarow, *J. Biol. Chem.* **168**, 705-10 (1947); Anthony J. Glazko and Loretta M. Wolf, *Arch. Biochem.* **21**, 241-2 (1949).

[95] Ruth Abbott Kaselis, Wladimir Liebermann, William Seaman, J. P. Sickels, E. I. Stearns, and J. T. Woods, *Anal. Chem.* **23**, 746-51 (1951).

[96] W. Jacobson and Delia M. Simpson, *Biochem. J.* **40**, 3-9 (1946); G. G. Villela, *O Hospital* **30**, 755-8 (1946); E. L. Whittle, B. L. O'Dell, J. M. Vandenbelt, and J. J. Pfiffner, *J. Am. Chem. Soc.* **69**, 1786-92 (1947); Gilberto Guimarães Villela, *Rev. brasil. biol.* **8**, 229-30 (1948); B. de Lerma, A. Colarusso and P. Bona, *Boll. soc. ital. biol. sper.* **24**, 1198-1200 (1948); N. A. Andreeva and V. N. Bukin, *Doklady Akad. Nauk.* (USSR) **64**, 95-8 (1949).

[97] V. Allfrey, L. J. Tepley, C. Geffen, and C. G. King, *J. Biol. Chem.* **178**, 465-81 (1949).

bacillus leichmanii is also used[98] with thiomalic or thiolactic acid as antioxidant.

Samples—*Lettuce, carrots, potato, beef muscle.* Grind a weighed sample with water for 5 minutes in a blender. Dilute so that the suspension contains 0.5 gram per ml., except in the case of beef muscle where it should be 0.4 gram per ml. Heat at 100° for 15 minutes and filter. Use a 10-ml. aliquot and treat by the procedure which allows for interfering pigments.

Grain, plants. Grind 5 grams of grain or 10 grams of green plants with sand and treat with 75 ml. of 40 per cent fresh metaphosphoric acid solution. After 45 minutes on a steam bath, cool, dilute to 100 ml., and filter. Boil and stir 50 ml. of filtrate for 5 minutes with 100 mg. of activated carbon which has been refluxed with 10 per cent aqueous aniline for 1 hour, filtered, washed, and dried at 40°. Filter by suction, wash, and discard the filtrate. Elute the vitamin from the carbon with 70 ml. of 1 volume of 1:2 ammonium hydroxide and 3 volumes of ethanol. Treat in 5 portions at 60-70°. Evaporate the extract to about 15 ml. and adjust to approximately pH 3 with 1:3 acetic acid. Add 4 per cent potassium permanganate solution until excess is present and after 10 minutes decolorize with 3 per cent hydrogen peroxide added dropwise. Adjust the pH to 4-4.5 and filter for examination by fluorescence.

Yeast, liver. Grind 2 grams of yeast or liver as above up to ". . . dilute to 100 ml. and filter." Adjust to pH 4.7 by dissolving sodium acetate in the sample and add takadiastase or equivalent. Store for 12 hours at 40-45°and adjust the pH to 3. Proceed from "Boil and stir 50 ml. of filtrate . . ." but allow for dilution by the enzyme preparation.

Pharmaceutical preparations. Dissolve a sample of about 0.3 gram in about 25 ml. of 0.4 per cent sodium hydroxide solution and dilute to 500 ml. Dilute a 10-ml. aliquot with 50 ml. of 1:10 hydrochloric acid and make to 100 ml.

Add about 60 ml. of the acid solution to 5 ml. of zinc amalgam and shake mechanically for 30 minutes. Pipet 10 ml. of the reduced solution into each of two 100-ml. low-actinic flasks. Add 5 ml. of 1:10

98 H. T. Thompson, L. S. Dietrich, and C. A. Elvehjem, *Ibid.* **184**, 175-80 (1950); Helen R. Skeggs, Helga M. Nepple, Katherine A. Valentik, Jesse W. Huff, and Lemuel D. Wright, *Ibid.* **184**, 211-21 (1950).

hydrochloric acid and 35 ml. of distilled water to one; and 10 ml. of acid and 30 ml. of water to the other. Also prepare a reagent blank. Develop by coupling.

According to the U.S.P., dissolve a sample containing 0.05 gram of folic acid in 0.4 per cent sodium hydroxide solution and dilute to 50 ml. with the same reagent. Mix 1 ml. of this solution, 75 ml. of water, and 18 ml. of 1:3 hydrochloric acid. Add 1 ml. of 0.5 per cent aqueous gelatine solution preserved with 0.1 per cent of benzoic acid. Dilute to 100 ml. Mix 75 ml. of this solution with 0.5 gram of zinc dust and shake occasionally for 15 minutes. Filter, rejecting at least the first 10 ml. of filtrate. Develop by coupling.

Procedure—*By coupling.* To develop 2 ml. of filtrate, mix with 5 ml. of water, add 0.7 ml. of 1:3 hydrochloric acid, and mix with 1 ml. of 0.1 per cent sodium nitrite solution. Mix and, after 3 minutes, add 1 ml. of 0.5 per cent ammonium sulfamate solution. After 2 minutes add 1 ml. of 0.1 per cent solution of N-(1-naphthyl)ethylenediamine dihydrochloride. Dilute with water to 10 ml. and mix. Read at 550 mμ after 5 minutes against a reagent blank. Interpret in terms of a curve prepared with *p*-aminobenzoic acid, which will not require the reduction step.

Correct for free *p*-aminobenzoic acid in the sample by a determination as follows. Dilute 2 ml. of the solution prepared by the USP method through "Dilute to 100 ml." to about 5 ml. Add 0.75 ml. of 1:3 hydrochloric acid. Continue in the above procedure by coupling from ". . . mix with 1 ml. of 0.1 per cent sodium nitrite solution."

Subtract the correction from the first reading, both in terms of *p*-aminobenzoic acid, and multiply by 3.22 to convert to folic acid.

An alternative, not U.S.P., is to read against a curve prepared from *p*-aminobenzoylglutamic acid. In that case multiply the result by 1.658 to convert to folic acid.

By fluorescence. Prepare an acetate buffer, pH 3.95, by adding 500 ml. of 1:2.5 acetic acid to 105 ml. of 20 per cent sodium hydroxide solution and diluting to 1 liter. This is subsequently referred to as the acetate buffer and a 1:9 dilution, that is 0.25 *M*, as the dilute buffer.

Pigments altered by permanganate oxidation absent. To 10 ml. of neutral, unbuffered solution containing up to 0.01 mg. of pteroyl glutamic acid per ml., add 0.1 ml. of the acetate buffer. The pH should be 3.9-4.1. Measure the background fluorescence of the solution against 0.1 mg. of quinine sulfate in 1:360 sulfuric acid diluted as necessary.

As filters use those for thiochrome and related substances,[99] Corning 5860 at 365 mμ as primary filter, and Corning 4308 and Corning 3389 for 470 mμ in that order with the latter facing the phototube. Add 0.05 ml. of 4 per cent potassium permanganate solution to the buffered solution and, after 5 minutes, add 0.1 ml. of 3 per cent hydrogen peroxide prepared by 1:9 dilution of the 30 per cent grade. The final pH should be between 4.1 and 4.4. Stir gently for 2-3 minutes and correspondingly determine the intensity of the fluorescence. Compare with standards applied to the same reference solution.

Interfering pigments present. Under these circumstances, the oxidation product of pteroylglutamic acid must be isolated by sorption on Florisil at pH 4. Prepare 60-100 mesh Florisil by suspending 500 grams in 2 liters of 4 per cent sodium tetraborate solution and boil for 30 minutes. Decant and repeat the process. Wash the Florisil and resuspend in 2 liters of the dilute acetate buffer. Boil for 30 minutes and filter. Wash with 10 liters of the dilute acetate buffer and air-dry at room temperature. As sorption column use a 5 × 120 mm. Pyrex tube with a bell-shaped reservoir at the top and drawn down to 1 mm. at the bottom. Prewash 10 cm. of the Florisil in this with the acetate buffer.

To a volume of solution containing up to 0.25 mg. of test substance, add 0.1 ml. of the acetate buffer and dilute to a volume of 10 ml. with water. The pH of the solution should be about 4. Add 0.05 ml. of 4 per cent potassium permanganate solution. If citrate, oxalate, or other oxidizable substances are present in high concentrations, add more permanganate to keep the solution a definite red during the oxidation. After 5 minutes add 0.1 ml. of 3 per cent hydrogen peroxide solution.

If the permanganate causes precipitation in the solution which does not dissolve on treatment with hydrogen peroxide, centrifuge before sorption. Pass through the Florisil in the column. After passing the solution through the column, wash the Florisil with five 10-ml. portions of dilute acetate buffer. To elute, pour four 5-ml. portions of boiling 4 per cent sodium tetraborate solution through the column. Apply mild suction to get an elution rate of 15-20 drops per minute. Adjust the pH of the eluate to 4-4.5 by the addition of about 1.8 ml. of 1:5 hydrochloric acid and dilute to 25 ml.

Add 0.1 ml. of acetate buffer solution to duplicate 10-ml. aliquots of eluate. Determine the intensity of fluorescence of 1 ml. of each of

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[99] Oliver H. Lowry, *Ibid.* 173, 677-82 (1948).

these samples before and after the addition of 0.1 ml. of 40 per cent sodium hydroxide solution. Read as for pigments altered by permanganate absent.

1-Aminoisovaleric Acid, Valine

To estimate valine it may be treated with triketohydrindene hydrate, ninhydrin, in neutral or slightly acid solution to produce an aldehyde with one less carbon atom.[100] The course of the reaction is not as definite as may be desired but use of *peri*-naphthindan-2,3,4-trione hydrate gives the reaction quantitatively.[110] It is also quantitative with aspartic acid, leucine, and phenylaminoacetic acid.

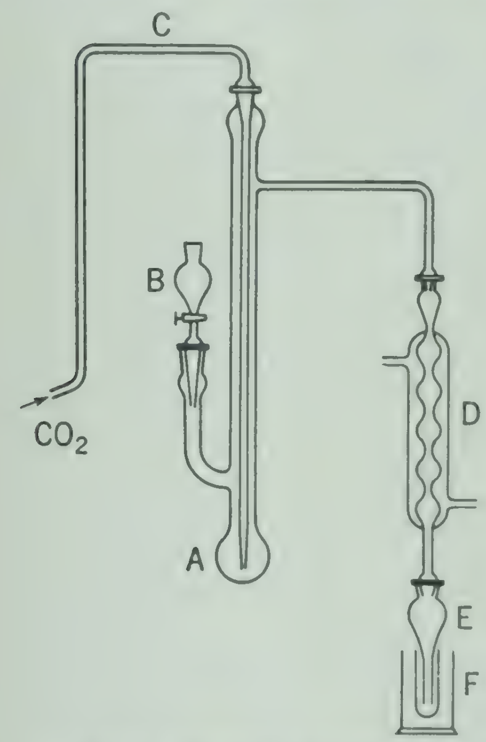

Fig. 9. Distillation apparatus

Procedure—As buffer for pH 4.7 grind together 17.65 grams of sodium citrate dihydrate and 8.4 grams of citric acid monohydrate. Transfer 10 mg. to the 10 ml. distilling flask of the apparatus shown in Figure 9. Add 1 ml. of the sample solution containing about 0.25 mg. of valine and 1 ml. of fresh 0.1 per cent solution of *peri*-naphthindan-2,3,4-trione. Pass carbon dioxide through for 2 minutes, then distil over half the contents. The solution is first orange, then red, finally pink. Add 4 ml. of water through B during the distillation and dilute the distillate to 10 ml.

Treat an amount of distillate expected to contain about 0.03 mg. of aldehyde with 2 ml. of exactly 10.5 *N* sodium hydroxide solution and 1 ml. of fresh 0.64 per cent salicylic aldehyde in absolute ethanol. Incubate at 50° for 70 minutes and cool for 10 minutes. Read against a reagent blank. When other aldehydes are developed, use the correspondingly different transmittance curve.

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[100] K. A. J. Wretlind, *Acta physiol. Scand.* 3, 329-34 (1942).

[101] Radwan Moubasher and William Ibrahim Awad, *J. Biol. Chem.* 179, 915-20 (1949).

ORNITHINE, α-δ-DIAMINOVALERIC ACID, 2,5-DIAMINOPENTANOIC ACID

Ornithine is determined as proline by a method described under that compound (page 166).

4-CARBAMIDO-1-AMINOVALERIC ACID CITRULLINE

Citrulline, in strongly acid solution, reacts with diacetylmonoxime to give a reddish yellow color.[102] Allantoin and urea vitiate results, by producing colors which are more yellowish. In a modification of the reaction, the preceding steps are followed by oxidation to give a red color.[103] The reaction is not specific but is given by all compounds having the general formula $RNHCONH_2$ and by some of those having the structure $R_1NHCONHR_2$. Among known tissue constituents the compounds other than citrulline which react are urea, allantoin, proteins, and higher protein derivatives such as peptone. Urea can be removed by urease but commercial preparations of urease often contain allantoic acid and canavanine, resulting in a large and incorrect blank unless they are removed by dialysis.[104] Allantoin and other plasma extractives can be eliminated by elution. Citrulline is sorbed on Amberlite at pH 6-7 and allantoin is not. By difference in chomagenic material before and after sorption a measure of citrulline is obtained.

There is no color reaction under the conditions of this method without the oxidation step by glutathione, ergothioneine, glutamyltyrosine, glycylglycine, proline, histidine, carnosine, anserine, glutamine, hippuric acid, *p*-aminohippuric acid, benzimidazole, urethane, uric acid, and allantoic acid. If heating is prolonged beyond 10 minutes, the following give interfering colors with the reagent: barbituric acid, phenobarbital, nembutal, caffeine, and hydantoin. Protein yields a pink color easily distinguishable from that of citrulline. Alloxan gives a faint yellow color but is not sorbed on Amberlite. Alloxantin and parabanic acid do not interfere due to their inability to sorb on Amberlite. Methylurea and phenylurea yield colors indistinguishable from that of citrulline. They interfere appreciably and are only par-

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102 W. R. Fearon, *Biochem. J.* **33**, 902 (1939); Reginald M. Archibald, *J. Biol. Chem.* **156**, 121-42 (1944); A. F. Lazarev, *Biokhimiya* **15**, 401-7 (1950).

103 Allan G. Gornall and Andrew Hunter, *Biochem. J.* **35**, 650-8 (1941).

104 R. M. Archibald and P. B. Hamilton, *J. Biol. Chem.* **150**, 155-8 (1943).

tially removed by the sorption process. Thymol interferes, cresol less, and phenol least.

After oxidation, maximum absorption occurs at 475 mμ. α and β-carbamidopropionic acids, ϵ-carbamidohexoic acid, and 3-carbamido-2-piperidone give the same maximum. Proteins are removed by the usual precipitants. Allantoin does not occur in human tissue but is present in animal tissue and in serum. Young chick skin procollagen, to which known amounts of tryptophan have been added, is suitable for preparation of a standard curve.[105] For 0.5-2.5 mg. of citrulline per 100 ml., Beer's law applies. The method is accurate to ±2 per cent.

Samples—*Plasma.* To 4 ml. of plasma, add 0.56 ml. of a 2 per cent solution of dialyzed urease in 2.6 per cent potassium cyanide solution, buffered with monobasic sodium phosphate to pH 7.2. Allow to stand at room temperature for 20 minutes. Incubate plasma from uremic patients for 1 hour. Dialyze for 2 hours against 10 ml. of 1:1500 sulfuric acid.[106] Develop without oxidation.

For preparation of a blank set up a sorption column. Place 2 inches of Amberlite IR-100 in the bottom of the column. Flush the column successively with the following at the rate of 3 drops per 2 seconds: 10 ml. of 10 per cent sodium chloride solution, 5 ml. of water, 10 ml. of concentrated hydrochloric acid, and 25 ml. of water. Dry with 5 ml. of alcohol, 5 ml. of ether, and a current of air. The column can be used for about 10 determinations and must then be prepared anew.

Reserve 4 ml. of dialyzate for the procedure. Adjust the remaining dialyzate to pH 6-7 with 72 per cent sodium hydroxide solution and pass through the column to remove citrulline. Sample and blank are for development without oxidation.

Tissue extracts. To destroy urea, treat 3 ml. of extract with 0.3 ml. of an acetate buffer for pH 5.0 (Vol. I, page 176), and 0.3 ml. of purified urease solution. Mix, add 1 ml. of 50 percent trichloroacetic acid, and heat for 2-3 minutes in boiling water to precipitate proteins. Cool, dilute to 15 ml., mix, let stand for a half hour, and filter. Use the filtrate as sample for development by oxidation.

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[105] V. N. Orekhovich and A. A. Tustanovskii, *Doklady Akad. Nauk, USSR* 67, 333-6 (1949).

[106] P. B. Hamilton and Reginald M. Archibald, *Ind. Eng. Chem., Anal. Ed.* 16, 136-7 (1944).

Procedure—*Without oxidation.* Mix 4 ml. of the dialyzate, 2 ml. of a 1:3 sulfuric-phosphoric acid solution, and 0.25 ml. of 3 per cent solution of diacetylmonoxime. Similarly treat 4 ml. of the material from which citrulline has been removed as the blank. Cap the tubes with glass marbles and heat in boiling water in the dark for 10 minutes. Cool in a water bath in a covered container. Approximately 10 minutes after the heating period, read at 490 mμ and subtract the blank. Protect the tubes from light during the period before readings.

With oxidation. To 7 ml. of protein-free filtrate containing 0.05-0.07 mg. of citrulline, add 4 ml. of concentrated hydrochloric acid and 0.5 ml. of a 3 per cent solution of diacetyl monoxime. Mix and immerse the filled portion of the tube in gently boiling water, keeping the upper part of the tube as cool as possible by means of a cover through which the tube is inserted. Put a funnel in the tube to serve as condenser and leave in the bath just 9 minutes. Remove and let cool for a few minutes to about 65°. Add 1 drop of a 1 per cent potassium persulfate solution and read against a reagent blank at 475 mμ. The color deepens gradually. When it no longer appears to change, add another drop of persulfate solution. If the color deepens, add a third drop. Take the reading at the maximum color.

Guanidine-1-aminovaleric Acid, Arginine

The reaction product of arginine with hypohalite develops a red color with α-naphthol. Hypochlorite develops the color slowly, hypobromite instantly, but deterioration starts quickly unless interrupted by the addition of urea.[107] Careful control of the amount of hypobromite added is essential for maximum color development. Too much will decrease the color.

The color is given according to molar concentration by arginine and methyl guanidine. Glycocyamine gives 70 per cent of the color expected

[107] Shoyo Sakaguchi, *J. Biochem. (Japan)* **5**, 25 (1925); *Ibid.* **38**, 91 (1951); C. J. Weber, *J. Biol. Chem.* **86**, 217-23 (1930); *Ibid.* **88**, 353-9 (1930); Erik Jorpes and Sigurd Thorén, *Biochem. J.* **26**, 1504-6 (1932); Reginald B. Fisher and Arthur E. Wilhelmi, *Ibid.* **32**, 606-8 (1938); Lloyd E. Thomas, Janet K. Ingalls and James Murray Luck, *J. Biol. Chem.* **129**, 263-71 (1939); Jacob W. Dubnoff, *Ibid.* **141**, 711-6 (1941); D. M. Doty, *Ind. Eng. Chem., Anal. Ed.* **13**, 169-72 (1941); Erwin Brand and Beatrice Kassell, *J. Biol. Chem.* **145**, 359-64 (1942); Herbert T. MacPherson, *Biochem. J.* **36**, 59-63 (1942); E. Rauterberg, *Die Chemie* **56**, 91-2 (1943); *Z. Ver. deut. Chem., Bieh.* **48**, 121-2 (1944); D. Vincent and P. Brygoo, *Bull. soc. chim. biol.* **28**, 43-8 (1946); J. W. Keyser, *Biochem. J.* **43**, 488-91 (1948).

from the molar concentration due to formation of an inner salt. Creatine, creatinine, urea, glycocyamidine, guanidine, and dimethylguanidine do not give the color reaction. Development of the sample specified is affected by the presence of proteins, 3 mg. of ammonium salts, 0.25 mg. of histidine hydrochloride, 0.4 mg. of tyrosine or tryptophane, glycocyamine, methylguanidine, 1 mg. of creatine, and 0.1 gram of urea.

In general, amino acids interfere by reacting with the α-naphthol to form colorless compounds and thus reduce the amount of color produced by arginine. The use of large amounts of α-naphthol can prevent this, but too large an excess must be avoided or a yellow color results. It is difficult, if not impossible, to completely screen this out photometrically. The interfering color from α-naphthol and hypobromite can be eliminated by use of 1-naphthol-8-sulfonic acid.[108]

Interfering glycocyamine is separated by sorbing the arginine on Permutit and later elution with 3 per cent sodium chloride solution. The amino acids obtained by dissolving a phosphotungstic acid precipitate in alkali may also be used as sample without interference by 1 per cent of sodium phosphotungstate. The color is greatly influenced by temperature. Unless the reagents are cold, a much fainter color is obtained. The color does not follow Beer's law over a long range. The method will estimate 0.05-0.005 mg. of arginine.

Oxine, 8-hydroxyquinoline, with its greater stability, is an appropriate substitute for α-naphthol.[109] Ammonia may then be 50 per cent greater than the arginine without serious interference. The violet color of substances having the configuration $NH{:}C(NH_2)NHR$ when heated with diacetyl in alkaline solution has also been applied to estimation of arginine.[110]

Sample—*Protein.* Hydrolyze 10–40 mg. of finely powdered protein of known moisture content with 3 ml. of 1:1 hydrochloric acid and 1 drop of octanol in an oil bath at 130-140° for 16 hours. Almost neutralize with 6.8 ml. of 10 per cent sodium hydroxide solution with cooling and dilute with water so that 1 ml. contains about 0.02 mg. of arginine for development with hypobromite and α-naphthol.

[108] H. Kraut, E. v. Schrader Bielstein, and M. Weber, *Hoppe Seyler's Z. physiol. Chem.* **286**, 248-56 (1951).

[109] Shoyo Sakaguchi, *Japan Med. J.* **1**, 278-81 (1948); *J. Biochem. (Japan)* **37**, 231-6 (1950).

[110] Konrad Lang, *Z. physiol. Chem.* **208**, 273-80 (1932).

Corn. Preparation of the appropriate solution is described under histidine (page 140).

Amino acid mixtures. Dissolve a sample expected to contain about 1-2 mg. of arginine in water containing 0.5 ml. of concentrated hydrochloric acid. Then dilute to 100 ml. with water for development with hypobromite and α-naphthol.

Beer and wort. See under tyrosine (page 126).

Urine. Dilute 5-10 times with water. Pass 5 ml. through a Permutit column of appropriate size. Rinse out the the residual glucosamine with 5 ml. of 0.3 per cent sodium chloride solution. Reserve the combined filtrates which contain all of the glucosamine for analysis for it. Elute the arginine with 10 ml. of 3 per cent sodium chloride solution. Dilute the filtrate to 10 ml. for development with hypobromite and α-naphthol. In this separation by Permutit, the salt concentration should not exceed 0.5 per cent. With not over 0.02 mg. of either arginine or glucosamine per ml. it may be 1 per cent.

Blood. Dissolved arginine. Use a filtrate deproteinized with phosphotungstic acid at 1:10 dilution. Heat coagulation of proteins is an alternative. If glucosamine is present separate arginine by elution as described under urine. Develop by hypobromite and α-naphthol.

Blood protein. Boil 45 ml. of serum or plasma with 750 ml. of 60 per cent ethanol, 50 ml. of saturated ammonium sulfate solution, and 50 ml. of molar acetate buffer for pH 4.6. Filter the protein precipitate, wash with boiling water, then with hot ethanol, finally with ether. Dry and hydrolyze as described for proteins.

Tissue. Prepare a suspension containing 1 gram of fresh tissue per 40 ml. of liquid. Adjust the pH to 6, immerse in boiling water for 10 minutes, cool, and filter. Develop an aliquot with hypobromite and α-naphthol.

Liver. Boil 20 grams of liver powder with 1500 ml. of 60 per cent ethanol, 100 ml. of saturated ammonium sulfate solution, and 100 ml. of molar acetate buffer for pH 4.6. Filter, wash successively with boiling water, hot ethanol, and ether. Dry and hydrolyze as described for proteins.

Procedure—*By hypobromite and α-naphthol.* Dilute a sample containing 0.04-0.4 mg. of arginine to 10 ml. Make alkaline to litmus with 10 per cent potassium hydroxide solution and add 1 ml. excess. Add 2 ml. of 0.1 per cent α-naphthol in 50 per cent ethanol and 1 ml. of 40 per cent urea solution. Mix and cool to room temperature. Add

1 ml. of a solution of 2 grams of bromine per 100 ml. of 5 per cent potassium hydroxide solution. Mix and after about 2 minutes add 1 ml. of 40 per cent urea solution. Add 1 ml. more of the hypobromite solution and dilute to 25 ml. Read at 530 mμ after 10 minutes. If the sample is protein, obtain the apparent content of arginine in several different aliquots of varying size and plot the apparent arginine content against the amount of protein present. Extrapolate to zero protein content to get the corrected arginine content.

An alternative technic[111] is to chill a 5-ml. sample to 0° in the cuvet. All reagents are also cooled to 0°. Add 1 ml. of 10 per cent sodium hydroxide solution and exactly 1 ml. of 0.02 per cent α-naphthol solution. Exactly 15 seconds later add 0.02 ml. of hypobromite, prepared by reaction of 2 grams of bromine with 100 ml. of 5 per cent sodium hydroxide. Add 1 ml. of 40 per cent urea solution. Read at 500 mμ against a reagent blank 7 minutes and 10 minutes after the sodium hypobromite solution was added.

By hypobromite and oxine. Prechill all solutions to approximately zero as in the preceding technic. Prepare the 0.02 per cent oxine reagent by diluting 0.2 per cent ethanolic solution of oxine with water.

Add 1 ml. of oxine solution and 1 ml. of 10 per cent sodium hydroxide solution to 5-ml. portions of sample solution containing not over 0.02 mg. of arginine. As in the previous method add to successive tubes 1-5 drops of 2 per cent bromine in 5 per cent sodium hydroxide solution. Shake and after 20 seconds add 1 ml. of 40 per cent urea solution. Dilute to 10 ml. with ice-cold water exactly 1 minute after the urea solution is added. This stabilizes the color for about 30 minutes at 0°. Read at 550 mμ against a reagent blank.

1-Aminocaproic Acid, Isoleucine

Some amino acids can be determined after hydrolysis of proteins, by conversion to hydroxy acids and oxidation to the corresponding aldehyde or ketone. Methyl ethyl ketone, obtained from isoleucine, is determined by reaction with vanillin.[112] Beer's law applies over the range of 0.02-0.14 mg. of ketone per ml. The colorimetric determination is accurate to about ±6 per cent, the overall determination including

[111] Ved. Vrat, *Permanente Foundation (Oakland, Calif.) Med. Bull.* 10, 177-82 (1952).

[112] Anthony A. Albanese and Virginia Irby, *Arch. Biochem.* 17, 21-30 (1948).

oxidation, to about ±8 per cent. Other amino acids found not to interfere include arginine, histidine, methionine, cystine, tyrosine, tryptophan, phenylalanine, serine, threonine, leucine, valine, lysine, alanine, and glycine. Acetone, acetaldehyde, and formaldehyde do not interfere.

Sample—*Proteins.* Hydrolyze 50 grams of protein by refluxing with 250 ml. of constant-boiling hydrochloric acid—37 per cent, specific gravity 1.19—for 24 hours. The nitrogen content of the hydrolyzate, which can be determined by a micro Kjeldahl procedure, should be 10-30 mg. per ml. Concentrate the hydrolyzate *in vacuo* to remove excess acid and filter to remove coagulated humin. Sulfuric acid digests of protein are not suitable because, when excess acid is precipitated as the barium or calcium salt, some isoleucine will be removed by sorption.

To convert the amino acids in the hydrolyzate to hydroxy acids, transfer an aliquot containing 50-150 mg. of nitrogen in a volume not exceeding 10 ml. and dilute to 10 ml. if necessary. Add slowly with agitation under a fume hood 5 ml. of 1:18 sulfuric acid, then 2 ml. of 50 per cent sodium nitrite solution. Shake occasionally during an interval of 10-15 minutes or until excessive bubbling has ceased. Add 3 ml. of 15 per cent urea solution and heat gently to remove residual nitrogen oxides.

In order to convert the hydroxy acid formed from isoleucine to methyl ethyl ketone, transfer the solution to the closed dropping funnel A shown in Fig. 10. Rinse the container with about 10 ml. of 1:70 sulfuric acid and adjust the volume to roughly 30 ml.

To flask D add 40 ml. of an oxidizing solution consisting of a 1:1 mixture of 1:9 sulfuric acid and 2 per cent potassium dichromate solution. To flask H add 10 ml. of fresh 5 per cent sodium bisulfite solution. Heat the liquid in flask D to boiling and turn on the suction leading to flask H. Adjust the valve to give a partial vacuum which will cause the solution in flask H to rise to about 1 cm. above the column of glass beads in column G.

Allow the solution in A to run into the boiling oxidizing solution at a rate to deliver it in about 5 minutes. Distil over 20 ml. of solution, as measured by a mark on tube G. Discontinue heating and suction and wash down tube G with 10 ml. of water. Transfer the distillate and washings containing methyl ethyl ketone to a 50-ml. volumetric flask, dilute to volume, and use an aliquot as sample.

Procedure—Prepare a vanillin reagent by dissolving 250 mg. of vanillin in 2.5 ml. of absolute methanol and diluting to 25 ml. with concentrated hydrochloric acid. To 1 ml. of sample solution containing 0.02-0.12 mg. of methyl ethyl ketone, add 1 ml. of concentrated sulfuric acid and 1 ml. of fresh vanillin reagent solution. Mix, let stand for exactly 30 minutes, dilute to 5 ml., mix, and read against a reagent blank at 600 mμ.

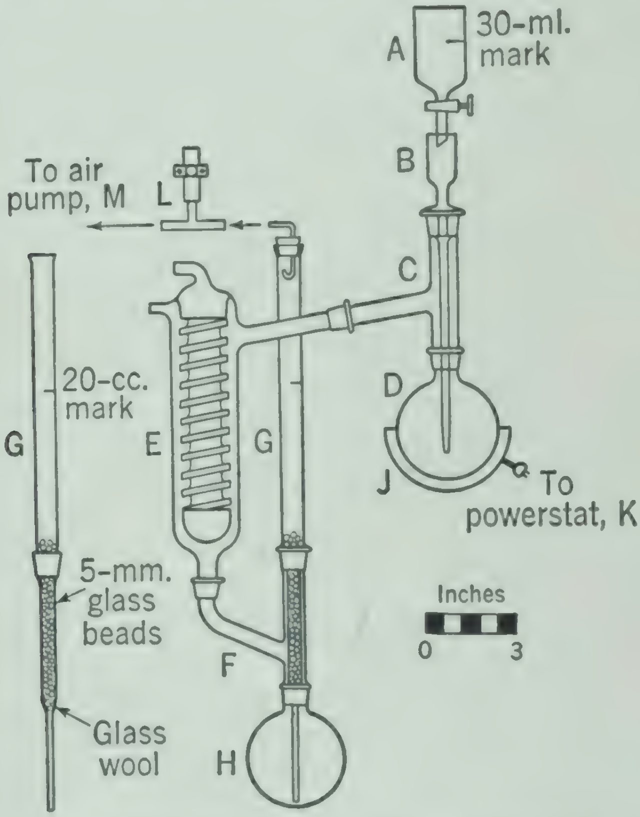

Fig. 10. Apparatus for oxidation of isoleucine to methylethyl ketone

Since 1 mg. of isoleucine is equivalent to 0.55 mg. of methyl ethyl ketone and oxidation of 1 mg. of isoleucine yields 0.095 mg. of methyl ethyl ketone, 1 mg. of the ketone is equivalent to 10.5 mg. of isoleucine.

1-Aminoisocaproic Acid, Leucine

Leucine is decomposed by *peri*-naphthindan-2,3,4-trione to give an aldehyde which may be estimated by reaction with alkaline salicyclic aldehyde. For details see valine (page 152).

1,5-Diaminocaproic Acid, Lysine

Lysine, after treatment with an oxidizing agent, gives a blue color with the phosphomolybdic-phosphotungstic acid reagent. Other amino acids which give the color include serine, threonine, phenylalanine, tyrosine, tryptophan, cystine, methionine, proline, hydroxyproline, leucine, histidine, and ornithine. Lysine can be separated from most interfering amino acids present in protein hydrolyzates by a sorptive column. This method has been used for separation of lysine and histidine, with lysine determined after correction by use of standard curves for both lysine and histidine.[113] In another method lysine and arginine are separated from other amino acids by sorption; after treatment with bromine or chlorine, arginine gives no color.[114] Chlorine is preferable to bromine. With its use accuracy to ±2 per cent may be obtained. Beer's law applies for concentrations of 0.001-0.03 mg. of lysine per ml.

Traces of histidine and ammonia are sorbed with the lysine and arginine, but the former are washed out of the sorptive column with aqueous pyridine. Arginine and lysine are eluted with sodium carbonate solution.

Sample—*Protein.* Wash 700 mg. of 40–60 mesh Decalso three times with 3 per cent sodium chloride solution, by decantation, then with water until free from chloride. Insert a small pad of cotton in a 3-inch, long-stemmed funnel constricted at the outlet. Pour in 25 ml. of water and immediately add the sorbent suspended in water. By tapping the funnel, an air-free column about 10 cm. long is obtained. Place a thin pad of cotton on top of the column and pour out excess water by tilting the funnel. Wash with 5 ml. of 10 per cent pyridine solution free from air bubbles. If the sample solution is not added at once, the column must be kept under liquid.

Hydrolyze a sample of protein by refluxing an amount containing 2-15 mg. of lysine with 10 ml. of 1:1 hydrochloric acid for 24 hours. Evaporate the hydrolyzate to dryness *in vacuo.* Take up the residue in exactly 10 ml. of 10 per cent pyridine solution and filter. Pass 1 ml. of the mixed amino acid solution containing 0.2-1.5 mg. of lysine through the sorption column until a depth of about 1 mm. of solution remains above the surface of the sorbent. Wash successively with three 1-ml.

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113 Andre C. Kibrick, *Arch. Biochem.* **20**, 22-4 (1949).

114 M. Boulet, J. A. Nelson, and W. D. MacFarlane, *Can. J. Research* **25A**, 540-7 (1947).

portions of 10 per cent pyridine solution, then with an additional 100 ml. of pyridine solution. Wash the pyridine out of the column with 4 ml. of water. Discard the filtrates and elute the lysine and arginine with 20 ml. of 2.12 per cent sodium carbonate solution. Adjust the pH to 3-4 with 1:5 hydrochloric acid and dilute to 50 ml. for use as sample.

Procedure—Prepare an acid chlorine solution by bubbling chlorine through a 20 per cent solution of barium chloride into 50 ml. of 1:5 hydrochloric acid for about 20 minutes. Mix 1 ml. of sample solution with 1 ml. of this and let stand for 5 minutes. Add 1 ml. of 5 per cent sodium arsenite solution to remove excess chlorine. Adjust the pH to 6.2-6.5 by addition of about 1.5 ml. of a buffer solution containing 2 grams of sodium citrate and 4 grams of trisodium phosphate dodecahydrate per 100 ml. Dilute to 9 ml., mix, and place in a water-bath at 90° for a few minutes. Add 1 ml. of phosphotungstic-phosphomolybdic acid reagent (Vol. III, page 116) containing 100 grams of lithium sulfate monohydrate per liter, from a pipet delivering about 1 ml. in 15 seconds. Let the liquid run down the wall of the cylinder without shaking. Continue heating for 40 minutes and cool. Read at 660 mμ against a reagent blank.

Hydroxylysine

Hydroxylysine, separated from interfering amino acids, is oxidized to form aldehyde with periodic acid for estimation.[115] Follow the procedure described under serine (page 119) for development without distillation.

Aminosuccinic Acid, Aspartic Acid

Aspartic acid is determined in protein hydrolyzates or other amino acid mixtures by conversion to fumaric acid, after which this is precipitated as the copper-pyridyl complex. The combined copper is determined colorimetrically.[116] Asparagine interferes. Biological fluids cannot be used because of possible interference by fumaric acid. The method, applicable to a minimum of 0.5 mg. of aspartic acid, is accurate to about ±5 per cent.

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[115] P. Desnuelle and S. Antonin, *Biochem. et Biophys. Acta* 1, 50-60 (1947).

[116] Felix Friedberg and Lawrence M. Marshall, *Proc. Soc. Exptl. Biol. Med.* 74, 446-8 (1950).

In the absence of serine, the method is to determine aspartic acid, serine, and alanine by conversion to the corresponding hydroxy acids, oxidation to aldehydes, and determination of the latter with sodium nitroprusside and piperazine.[117] After alanine has been determined separately, the amount of serine and aspartic acid are calculated by difference. The method is accurate to about ±6 per cent.

Aspartic acid is decomposed by *peri*-naphthindan-2,3,4-trione to give an aldehyde which may be estimated by reaction with salicylic aldehyde and alkali. For details see valine.

Sample—*Proteins.* Reflux for 20 hours with 10 times the weight of 1:1 hydrochloric acid. To 1 ml. of protein hydrolyzate or other aqueous solution containing 0.6-1.4 mg. of aspartic acid add alternately dropwise, 0.25 ml. each of dimethyl sulfate and 33 per cent sodium hydroxide solution, over a one-hour period, with mechanical shaking. Place in a refrigerator overnight, then adjust to about pH 2 with 1:2 sulfuric acid.

Silica gel is next required and is prepared as follows.[118] Let commercial granular sodium silicate stand for 10-24 hours covered with water. Filter and mix 500 grams with 2 liters of water. Add concentrated hydrochloric acid to a color change with methyl orange, then 150-200 ml. more. Let stand for 3 hours and filter by suction.

Suspend the gel in 3 liters of concentrated hydrochloric acid overnight, filter by suction, and wash with 5 liters of 1:1 hydrochloric acid, 10 liters of water, 10 liters of ethanol, and finally with 5 liters of dry ether. Dry in a warm current of air, let stand for 2 weeks, suspend in 4 liters of concentrated hydrochloric acid, and let stand overnight. Filter by suction, wash with 5 liters of 1:1 hydrochloric acid, 50 liters of water, and 10 liters of absolute ethanol, containing 1 per cent of 1:1.5 sulfuric acid. Wash with ether. Dry in air. Before using dry for at least 24 hours over phosphorus pentoxide. Grind to pass through a 90-mesh sieve.

Mix 1 gram of the 90-mesh silica gel with the sample and disperse in 15 ml. of 1:3 butanol:chloroform mixture. Filter and wash with 20 ml. of 1:3 butanol:chloroform. Dry the combined filtrate and washings in a current of air at room temperature. Dissolve this dry residue of

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117 C. Fromageot and P. Heitz, *Mikrochim. Acta* **3**, 52-67 (1938); Pierre Desnuelle, *Enzymologie* **5**, 37-43 (1938).

118 L. M. Marshall, J. M. Orten, and A. H. Smith, *J. Biol. Chem.* **179**, 1127-39 (1949).

fumaric acid in 1 ml. of water at 70° for development as copper diethyldithiocarbamate.

For development with sodium nitroprusside and piperizine, hydrolyze and convert the amino acids to hydroxy acids according to the method for alanine to "Shake and dilute to 100 ml." (page 116).

To an aliquot in a distilling flask add 10 ml. of 2 *N* phosphoric acid and enough 10 per cent manganous sulfate solution to give a 4 per cent solution of the latter. For example, add 20 ml. of manganous sulfate solution to a 50-ml. aliquot. Connect the flask for steam distillation and add 1 per cent potassium permanganate solution for 60 minutes at the rate of a drop every 3-4 seconds. Complete as in the method for alanine starting with "Continue boiling for 10 minutes" (page 117).

Subtract the value for alanine from that obtained in this method for alanine plus serine plus aspartic acid.

Procedure—Develop by the procedure for fumaric acid as copper diethyldithiocarbamate (Vol. III, page 381).

γ-Amino-α-(*o*-aminophenyl)glutaconic Acid, Kynurenine

The red dye formed by diazotized kynurenine with N-α-diethylpropylenediamine hydrochloride is applicable in the absence of *m*-, and *p*-aminobenzoic acids.[119] These interferences can be serious since *o*-aminobenzoic acid gives 9 times the intensity of color developed with kynurenine and *p*-aminobenzoic acid 17 times the intensity. A separation by extraction eliminates that difficulty.

Diazotized kynurenine develops a red-orange color with 1-naphthol, the maximum absorption being at 480 mμ.[120] Anthranilic acid gives a yellow-orange but can be separated by ether extraction. *p*-Aminobenzoic acid gives red-orange and sulfanic acid a pink color. There is no color from 5-hydroxyanthranilic acid, *p*-aminosalicylic acid, *o*-aminoacetophenone, or 3-hydroxy-*o*-aminoacetophenone. When 1-naphthol is replaced by 2-ethylaminoethyl-1-naphthylamine the maximum absorption for kynurenine is at 560 mμ.[1] The same color is given by aniline, anthranilic acid, 5-hydroxyanthranilic acid, *p*-aminosalicylic acid, and *o*- and *p*-aminoacetophenone. These interferences are extractable with butanol.

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[119] Joseph Tabone and Colette Magis, *Compt. rend.*, 221, 123-5 (1945); *Bull. soc. chim biol.* 28, 744-7 (1946); Colette Magis and Joseph Tabone, *Ibid.* 28, 747-50 (1946).

[120] Noboru Ito and Kunio Motizuki, *J. Nara Med. Assoc.* 1, 15-17 (1950).

Procedure—Acidify 5–10 ml. of sample solution, previously cooled in ice, with trichloroacetic acid. Add 0.5 ml. of a 1 per cent solution of sodium nitrite and stir for 3-4 minutes. Destroy excess nitrite by addition of 1 ml. of a 5 per cent solution of ammonium sulfamate and stir for 3-4 minutes. Keep in ice water during all of these treatments. Add 1 ml. of a 1 per cent solution of N-α-naphthyldiethylpropylenediamine.

When the maximum color is attained, extract it with 2-4 ml. of isoamyl alcohol and discard the aqueous phase. Shake the amyl-alcohol extract with an equal volume of 1:5 sulfuric acid. If the solution contains only kynurenine, the aqueous phase is red, the upper amyl-alcohol phase colorless. If only aminobenzoic acids are present, the upper phase is red, the lower phase colorless; if both compounds are present, both layers are red. Read the lower layer against a reagent blank.

Pyrrolidene-2-carboxylic Acid, Proline

Proline and ninhydrin form a red reaction product at pH 7.[121] Hydroxyproline gives a similar but not identical reaction product. At pH 1 proline forms a red water-insoluble reaction product with ninhydrin where lysine forms a black product and ornithine a red one.[122] Other amino acids do not interfere at pH 1.[123]

There is no interference with ornithine or proline at 515 mμ by 100 times the amount of leucine, isoleucine, serine, valine, threonine, glutamic acid, glycine, alanine, δ-aminovaleric acid, urea, creatine, creatinine, glycocyamine, glucosamine, ammonium chloride, or pyrrolidonecarboxylic acid. There is no interference by 10 times the amount of glutamine, histidine, cystine, methionine, tryptophan, arginine, tyrosine, hydroxyproline, aspartic acid, asparagine, phenylalanine, 1,4-diaminobutane, or 1,4-diaminopentane. There was interference by 10 times the amount of citrulline, cysteine, lysine and hydroxylysine. The interference of hydroxyproline is half that of proline. Apparent interference by arginine is probably due to an impurity.

The oxidation of proline or hydroxyproline with lead dioxide in the presence of copper sulfate yields products which react with *p*-di-

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121 W. Grassmann and K. von Arnheim, *Ann. Chem.* **509**, 288-303 (1934); *Ibid.* **519**, 192-208 (1935).

122 D. D. Van Slyke, R. T. Dillon, D. A. MacFadyen, and P. Hamilton, *J. Biol. Chem.* **141**, 627-69 (1941).

123 Francis P. Chinard, *J. Biol. Chem.* **199**, 91-5 (1952).

methylaminobenzaldehyde.[214] In this reaction, 1 mg. of hydroxyproline produces the same intensity of color as 10 mg. of proline. Hence the method is inapplicable if both are present. The oxidation products of both proline and hydroxyproline can be steam-distilled. Proline is also determined by hypochlorite oxidation as described under hydroxyproline.

Sample—*Casein.* Add 40 ml. of a saturated barium hydroxide solution to 2 grams of casein and autoclave for 3 hours at 150° and 70 pounds pressure. Add 10 ml. of 10 per cent sulfuric acid solution, cool, and filter. Wash the residue on the filter paper with water until the volume of the filtrate is 100 ml. Determine by dimethylaminobenzaldehyde.

Procedure—*By ninhydrin.* As reagent mix 4 ml. of 1:1 dilution of sirupy phosphoric acid and 6 ml. of glacial acetic acid. Add 0.25 gram of ninhydrin and heat to 70° to obtain complete solubility.

To 1 ml. of sample solution add 1 ml. of glacial acetic acid and 1 ml. of the reagent. Only silicone grease may be used on stopcocks. At the same time prepare a sample blank, using the acid mixture without ninhydrin. Also prepare a reagent blank.

Heat the sample and blanks at 100° for 60 minutes, with caps in place. Add 1 ml. of glacial acetic acid to each and cool to room temperature. Adjust the volume of each to 5 ml. with glacial acetic acid. Read within 1 hour. The wave lengths for the various amino acids are as follows:

Proline or ornithine	515 mμ
Hydroxylysine	450 mμ
Lysine	500 mμ
Cysteine	570 mμ

By dimethylaminobenzaldehyde. Neutralize a 5-ml. aliquot of filtrate to phenolphthalein with 4 per cent sodium hydroxide solution and follow with 5 ml. of water, 10 ml. of phosphate buffer for pH 8.7, and 1 gram of lead dioxide. Reflux for 30 minutes, cool, and filter. Wash the residue with water and dilute the filtrate to 200 ml. To a 5-ml. aliquot of this solution add 5 ml. of water, 1 ml. of 4 per cent solution of *p*-dimethylaminobenzaldehyde in ethanol and 1 ml. of 1:5 hydrochloric acid. Heat

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124 G. H. Guest, *Can. J. Research* **17B**, 143-4 (1939).

in boiling water for one minute and cool for 5 minutes under running water. Read at 520 mμ against a reagent blank.

4-Hydroxypyrolidene-2-carboxylic Acid, Hydroxyproline

The oxidation of hydroxyproline with hydrogen peroxide catalyzed by copper ion yields products which form an intense red color with *p*-dimethylaminobenzaldehyde.[125] The method has an accuracy of ±2 per cent. Tyrosine appears to be the only practical interfering substance when protein hydrolyzates are developed by this method. Its color can be differentiated from that of hydroxyproline by its bronze hue with maximum absorption at 500 mμ instead of 550 mμ, by its greater stability, and by its greater solubility in amyl alcohol. Pure tryptophan yields color corresponding to 0.7 per cent hydroxyproline, but does not really interfere because it is eliminated from proteins in the form of humin during acid hydrolysis. If large amounts of tryptophan are present but form small amounts of humin, add some glucose before hydrolysis to insure destruction of the tryptophan. So far as tyrosine interference is concerned, the best solution appears to be a separate determination and correction on the basis that it gives approximately 1.5 per cent of the color intensity of hydroxyproline. Proline gives less than 10 per cent of the color of hydroxyproline. Sodium hypobromite can also be used as the oxidizing agent, with subsequent decolorization with sodium thiosulfate.[126]

The color may also be developed as a stable red complex with isatin after the peroxide oxidation catalyzed by copper or cobalt,[127] but results tend to be low.[128] In an earlier version of the reaction,[129] hypochlorite was used to split off carbon dioxide and water from proline and hydroxyproline to give pyrroline and hydroxypyrroline. These are then steam-distilled for estimation. A subsequent reaction with *p*-dimethylaminobenzaldehyde gives the color due to both; that with isatin in dilute sulfuric acid, only the color due to hydroxypyrroline. Pyrroline was therefore estimated by difference. The color fades quickly. Determination of the hydroxyproline is more exact because the intensity of color

[125] Robert E. Neuman and Milan A. Logan, *J. Biol. Chem.* **184**, 299-306 (1950).

[126] Dieter Müting, *Hoppe-Seyler's Z. physiol. Chem.* **291**, 234-5 (1952).

[127] W. D. MacFarlane and G. H. Guest, *Canad. J. Res. Sect. B.* **17B**, 139-42 (1939).

[128] John Devine, *Biochem. J.* **35**, 433-45 (1941).

[129] K. Langheld, *Ber.* **42**, 2360-74 (1909); Konrad Lang, *Z. physiol. Chem.* **219**, 148-54 (1933).

is 8 times that developed from proline. Beer's law does not apply strictly to either reaction. In the absence of proline, the oxidation and development with dimethylaminobenzaldehyde are applicable to hydroxyproline.

Samples—*Protein.* Autoclave 50 mg. of protein with 1 ml. of 1:1 hydrochloric acid for 3 hours at 50 pounds pressure. Neutralize, dilute to an appropriate volume, and filter if necessary.

Procedure—To 1 ml. of solution containing 0.005–0.015 mg. of hydroxyproline, add 1 ml. each of 0.25 per cent copper sulfate solution, 10 per cent sodium hydroxide solution, and 6 per cent hydrogen peroxide solution. Mix, shake occasionally during 5 minutes, and place in a water bath at 80° for 5 minutes with shaking. The heating and shaking will destroy excess peroxide. Chill to 0° and add 4 ml. of 1:11 sulfuric acid with agitation. Add 2 ml. of 5 per cent *p*-dimethylaminobenzaldehyde in *n*-propanol. Place in a water bath at 70° for 10 minutes and cool under tap water. Read at 540 mμ against a reagent blank.

Canavanine

A solution of oxidized sodium nitroprusside reacts with canavanine, $H_2NC(:NH)\cdot NH\cdot O\text{-}CH_2CH_2CHNH_2COOH$, to give a ruby-red solution.[130] Strictly speaking the reaction is one for estimating the guanadino group, $NH_2C(:NH)NHOR$. The color only approximates conformity to Beer's law.

There is no appreciable color given with this reagent at pH 7.2 by acetone, acetoacetate, arginine, glycocyamine, creatine, creatinine, guanidine, and ammonium sulfate. Glutathione and cysteine yield a gray-purple but the color fades completely in 30 minutes. Thiourea, thiouracil, and sulfide give a blue color, but their absorption curves are so different from that of canavanine that interference is not likely. The color given by thiouracil develops rapidly but fades in 2 hours. Derivatives of hydroxyguanidine give an appreciable red color. The method is not applicable to urine and plasma because of the presence of an unidentified reducing substance which inhibits color development.

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[130] Matsunosuke Kitagawa, and Akishige Takani, *J. Biochem.* (Japan) 23, 181-5 (1936); Matsunosuke Kitagawa, *Ibid.* 24, 107-12 (1936); Reginald M. Archibald and Paul B. Hamilton, *J. Biol. Chem.* 150, 155-8 (1943); Reginald M. Archibald, *Ibid*, 165, 169-78 (1946).

Procedure—As an aquoprusside reagent mix 0.5 ml. of 20 per cent potassium carbonate solution and 0.4 ml. of 30 per cent hydrogen peroxide with 10 ml. of 2 per cent sodium nitroprusside solution. Allow to stand 30 minutes at room temperature before using and prepare fresh daily. As a molar phosphate buffer for pH 7.2, prepare a solution containing 3.45 per cent of monosodium phosphate monohydrate and 13.06 per cent of dipotassium phosphate.

To 1 ml. of neutral sample solution containing up to 0.25 mg. of canavanine, add 0.5 ml. of the phosphate buffer solution and 0.5 ml. of aquoprusside reagent. Mix thoroughly and store in the dark. After 2 hours read at 520 mμ against water and correct for a reagent blank. The readings may be delayed a further 6 hours without loss of color.

CHAPTER 4

PROTEINS [1]

Proteins consist of compounds of the amino groups of amino acids with carboxyl groups of such acids. The molecules are composed of many such acids both in number and often in structure. They vary in molecular size. Proteins are classified into several fractions of varying molecular weight and solubility in water, usually designated as globulins, albumins, fibrins, etc. Proteins are introduced at this point because of their close relation to the amino acids of the preceding chapter.

Many are determined as amino acids. Other methods of determination are as ammonia by Nessler's reagent, the biuret method with a copper reagent, the xanthoproteic acid reaction by nitration, etc., including several nephelometric methods.

As organized, total protein is followed by the various fractions, the total nitrogen, ammonia, then fractions which are determined after segregation from total protein.

Total Protein

The various forms of nitrogen, properly segregated, are oxidized to ammonia and determined by Nessler's reagent.[2] The degree of alkalinity of the final solution of the sample must be rigidly standardized (Vol. II, pages 814-19). Trouble with precipitation in the final solution is attributed to silica dissolved from the glass in digestions. Such precipitation is prevented by addtion of a protective colloid. Replacement of sodium hydroxide by lithium hydroxide is helpful. Because of the cloudiness which frequently develops with Nessler's reagent, a modified form gives nephelometric results. The reagent is sufficiently sensitive to produce a bluish white cloud with 1 ppm. of ammonia. The usual factor for protein is Nessler nitrogen times 6.25.

Freshly precipitated copper oxide is dissolved by an alkaline protein solution to give the biuret reaction, a violet color proportional to the

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[1] See Chapter 1, Volume III, for details of organization, condensation, etc.

[2] William S. Hoffman and B. Osgood, *J. Lab. Clin. Med.* **25**, 856-62 (1940).

amount of protein present.[3] This color is relatively stable and applicable to protein fractions. In practice addition of a copper sulfate solution to an alkaline solution is convenient. If the protein solution is neutral the alkalinity may be introduced with the biuret reagent, as by using 1 per cent copper sulfate pentahydrate solution containing 11.7 per cent of sodium hydroxide, or 0.1 per cent copper sulfate in 12.5 per cent sodium hydroxide solution.[4] It follows that this may be applied to a solution such as serum, to the precipitated and washed protein from serum, or to the fractions such as globulin and albumin into which the protein is fractionated.

Turbidity due to lipids is often eliminated by chilling and filtering.[5] Results agree with Kjeldahl determination within ±3 per cent. The determination of proteins by the biuret method is more accurate than by microkjeldahl or the tyrosine reaction.[6] A high proportion of non-peptide nitrogen in the protein tends toward low results.[7] Another reagent is a modified Fehling solution, 0.15 per cent of cupric sulfate pentahydrate, 3 per cent of sodium hydroxide, and 0.6 per cent of sodium potassium tartrate.[8] Lipids and ammonium sulfate must be absent. Yet another is 4.5 per cent of sodium potassium tartrate, 1.5 per cent

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[3] E. V. Dodonova and N. N. Ivanov, *Biokhimaya* **3**, 723-30 (1938); *J. Applied Chem.* (USSR) **12**, 1575-81 (1939); Marie A. Andresch, *Am. J. Clin. Path., Tech. Sect.* **8**, 43-6 (1944); Rubens Salomé Pereira, *Rev. faculdade med. vet., Univ. São Paulo* (Brazil) **2**, 257-73 (1944); J. L. Moglia, F. Vilallonga, and A. D. Marenzi, *Pubs. centro invest. tisiol.* (Buenos Aires) **9**, 133-43 (1945); Alma Hiller, Roger L. Greif, William W. Beckman, and John Plazin, *J. Biol. Chem.* **176**, 1421-9 (1948); Bertil Josephson and Hans Andurén, *Acta Paediat.* **38**, 335-9 (1949); Robert Ardry, *Ann. biol. clin.* (Paris) **7**, 407-15 (1949); J. P. Dustin, *Arch. intern physiol.* **57**, 95-7 (1949); Rolf Emmerich, *Z. ges. inn. Med.* **4**, 178-82 (1949); Karl Hinsberg and Jörn Gliess, *Klin. Wochschr.* **28**, 444-8 (1950); M. I. Plekhan, *Zhur. Obshcheĭ Khim.* **21**, 574-9 (1951).

[4] George R. Kingsley, *J. Biol. Chem.* **131**, 197-200 (1939; *J. Lab. Clin. Med.* **27**, 840-5 (1942); *J. Lab. Clin. Med.* **29**, 436-8 (1944); Lucille Book and Donald W. Bolin, *Proc. N. Dakota Acad. Sci.* **1**, 28-30 (1948).

[5] Robert N. Feinstein, *Anal. Chem.* **21**, 534 (1949).

[6] Rogerio F. Magalhães Gomes, *Arquiv. inst. biol. exército* (Rio de Janeíro) **7**, 199-206 (1946).

[7] P. Bandet and Cl. Giddey, *Helv. Chim. Acta* **31**, 1879-84 (1948).

[8] Allan G. Gornall, Charles J. Bardawill, and Maxima M. David, *J. Biol. Chem.* **177**, 751-66 (1948); Jorge Furio Frattini, *Rev. asoc. bioquim. argentina* **15**, 372-8 (1951); Ant. Heyrovsky and I. Sochorova, *Acta Med. Scand.* **141**, 65-9 (1951); Mavis Freeman, *Med. J. Australia* **1951**, II, 114-15; Cf. Max F. Jayle, G. Boussier, and J. Badin, *Bull. soc. chim. biol.* **33**, 881-2 (1951).

of cupric sulfate pentahydrate, 0.5 per cent of potassium iodide, and 0.3 per cent of sodium hydroxide.[9]

A variation of the method is to treat a solution of protein in trisodium phosphate solution with solid cupric phosphate. After filtration the copper is then complexed and read at 440 mμ.[10] In another variation, by use of ethylene glycol the precipitation of cupric hydroxide is prevented. Reading is then in the ultraviolet at 320 mμ.[11]

The proteins can be precipitated with 1-7 per cent perchloric acid.[12] The acid does not interfere with copper reduction. The reaction is also obtainable more slowly in alkaline solution with nickel nitrate or cobalt nitrate. Their maxima are in general at 660 and 480 mμ.[13]

Protein hydrolyzates are read as copper salts of amino acids and peptides in the ultraviolet at 235 mμ or as the copper alaninate at 620 mμ (page 110).[14] The copper salts of glycine and alanine approximate maxima at 235 mμ.[15] Values in the ultraviolet are over 100 times as sensitive when applied to protein hydrolyzates.[16] The maximum absorption is lower than 230 mμ for glycine and histidine and higher for cystine, tyrosine, hydroxyproline, and proline. Tryptophan is anomalous in giving very high values.

Dipeptides and tripeptides in general have no maximum down to 212-214 mμ. Where the amino acids form CuA_2, the peptides form CuP. Table 11 compares the readings with alanine.

Total protein in many samples is estimated by phosphotungstic-phosphomolybdic acid, usually known as the Folin phenol reagent.[17] Heating an alkaline solution of protein for a short time before adding the reagent gives an increase of intensity.[18] The reagent itself is decomposed by alkali so that this is preferable to simultaneous addition.

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[9] T. E. Weichselbaum, *Am. J. Clin. Path. Tech. Sect.* **10**, 40-9 (1946); cf. Marlowe Ditterbrandt, *Am. J. Clin. Path.* **18**, 439-41 (1948); Wilhelm Bitter, *Z. ges. inn. Med. u. Grenzgebeite* **7**, 978-81 (1952).

[10] Henry A. Stiff, Jr., *J. Biol. Chem.* **177**, 179-85 (1949).

[11] John W. Mehl, *Ibid.* **157**, 173-80 (1945).

[12] C. Neuberg, E. Strauss, and L. E. Lipkin, *Arch. Biochem.* **4**, 101-4 (1944).

[13] M. I. Plekhan and A. M. Karr, *Zhur. Obshcheĭ Khim.* **20**, 2105-10 (1950).

[14] Joseph R. Spies and Doris C. Chambers, *J. Biol. Chem.* **191**, 787-97 (1951).

[15] H. Ley and H. Hegge, *Ber.* **48**, 70-85 (1915).

[16] Joseph R. Spies, *J. Biol. Chem.* **195**, 65-74 (1952).

[17] Hsien Wu, *J. Biol. Chem.* **51**, 33-9 (1922); Schmorl W. Ling, *Ibid.* **69**, 397-401 (1926); Humberto Giovambattista and Raul Nico, *Rev. facultad. cienc. quím. Univ. nacl. La Plata* **15**, 7-11 (1940).

[18] David Pressman, *Ind. Eng. Chem., Anal. Ed.* **15**, 357-9 (1943).

Since this procedure is general for proteins, it follows that the technic is applicable to fractions isolated in various ways, providing only that interfering substances such as phenols are not present. Many proteins give a deeper color with the phenol reagent if a trace of copper sulfate is present.[19] This is not the case with free amino acids. The presence of copper results in color with biuret, oxamide, malonamide, and some peptides which give no color in its absence. This reaction is as sensitive as the Nessler reaction, yet requires no digestion. It is 10-20 times as sensitive as reading at 280 mμ. It is 100 times as sensitive as the biuret reaction.

TABLE 11. ALANINE EQUIVALENCE OF COPPER SALTS OF DIPEPTIDES AND TRIPEPTIDES MEASURED AT 230 mμ

Substance	Wave-length of Maximum Absorbancy	Molarity of Test Solutions × 10⁴	Alanine Equivalent of Test Solutions: Found	Alanine Equivalent of Test Solutions: Estimated if Amino Acids Were Free
	mμ		*per cent*	*per cent*
Alanine		3.0	100.0	100
Dialanylcystine	217-220	0.75	77.2	100
Alanylglycine	<213	1.5	79.5	100
Glycylalanine	<213	1.5	75.2	100
Glycylaminoisobutyric acid	<213	1.5	73.5	100
Glycylamino-*n*-butyric acid	<214	1.5	75.4	100
Glycyldehydrovaline	223-224	1.5	47.1	
Glycylsarcosine	216-220	1.5	18.7	
Glycylserine	<214	1.5	69.8	100
Sarcosylphenylalanine	222	1.5	68.7	100
Glycylalanylglycine	<213	1.5	84.6	150
Glycylglycylalanine	<212	1.5	85.8	150
Glycylglycylleucine	<214	1.5	84.8	150
Glycylleucylglycine	<213	1.5	89.0	150
Leucylglycylglycine	<213	1.5	87.2	150

Precipitation of the protein by phosphomolybdic acid is followed by reduction to the familiar molybdenum blue on the assumption that there was present in the precipitate a definite ratio of phosphomolybdic

[19] P. Mounier, *Bull. biol. pharm.* **1937**, 167-75; *Ibid.* **1939**, 81-95; Roger M. Herriott, *Proc. Soc. Exptl. Biol. Med.* **46**, 642-4 (1941); E. W. Sutherland, C. F. Cori, R. Haynes, and N. S. Olsen. *J. Biol. Chem.* **180**, 825-37 (1949); Oliver H. Lowry, Nira J. Rosebrough, A. Lewis Farr, and Rose J. Randall, *Ibid.* **193**, 265-75 (1951).

acid to protein.[20] Results by this indirect method agree with those by other accepted methods.

The reaction for arginine with α-naphthol when appropriately factored is a measure of proteins. Although the dispersion characteristics of different proteins differ, their arginine content appears to be relatively constant.[21] Similarly the histidine reaction with diazotized sulfanilic acid is applied.[22]

The xanthoproteic action of proteins with nitric acid to give a brown to purple color is applicable to estimation of total protein at 420 mμ.[23] It is sometimes referred to as the xanthoproteic index when applied to blood serum. Many structures nitrate under the conditions and therefore interfere. They include phenol, salicylic acid, aminopyrine, tyrosine, tryptophan, tyramine acid phosphate, acetyl salicylic acid, sulfanilamide, *p*-aminobenzoic acid, sulfadiazine, sulfapyridine, and sulfathiazol.

Sulfosalicylic acid is a suitable precipitant to use in nephelometric estimation of protein.[24] Under the conditions used, this does not precipitate amino acids, peptides, peptones, or urinary constituents. The turbidity decreases on standing so that after 10 minutes there is a variation of 5 per cent from the original values. Accuracy to 0.3 per cent is attainable with small volumes. In general the method is accurate to 5 per cent and agrees well with results by other methods. Various forms of permanent standards are available.[25] A special form of this reagent consists of 3 per cent of melted phenol, 3 per cent of 40 per cent

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[20] Burnham S. Walker and Henry J. Bakst, *J. Lab. Clin. Med.* **20**, 312-4 (1934).

[21] Schoyo Sakaguchi, *J. Biochem.* (Japan) **5**, 13-24 (1925); Anthony A. Albanese, Virginia Irby, and Barbara Saur, *J. Biol. Chem.* **166**, 231-7 (1936); Anthony A. Albanese, Barbara Saur, and Virginia Irby, *J. Lab. Clin. Med.* **32**, 296-9 (1947).

[22] N. N. Ivanov and E. V. Dodonova, *Doklady Vsesoyuz. Akad. Sel'skokhoz. Nauk. im. Lenina* **1940**, No. 23-4, 29-30.

[23] Jeáo Baptista dos Rios and Hans Schmidt, *O Hospital* **24**, 571-4 (1943); James F. Mezen and Roger S. Hubbard, *Am. J. Clin. Path.* **14**, 229-33 (1944).

[24] J. A. F. Pfeiffer, P. A. Kober and C. W. Field, *Proc. Soc. Exptl. Biol. Med.* **12**, 153-7 (1915); A. Sartory, R. Sartory, and J. Meyer, *Bull. biol. pharm.* **1937**, 503-6; Joseph M. Looney and Anna I. Walsh, *J. Biol. Chem.* **127**, 117-21 (1939); *Ibid.* **130**, 635-9 (1939); T. U. Marron, *Am. J. Clin. Path., Tech. Suppl.* **6**, 37-42 (1942); Harold B. Salt, *J. Lab. Clin. Med.* **35**, 976-82 (1950).

[25] E. J. King, *Biochem. J.* **48**, 50-1 (1951).

formaldehyde, and 3 per cent of sulfosalicylic acid in water.[26] When solutions of protein at 0.0015-0.0065 mg. per ml. in 0.06 per cent acetic acid are treated with a solution of potassium ethyltetrabromophenolphthalein, the protein is read at 600 mμ.[27]

Nephelometric examination of samples, with varying amounts of ammonium sulfate added, gives nephelometric values for the total protein, albumin, globulin, and fibrinogen fractions.[28] The optical properties of the solution vary with the rate of addition of ammonium sulfate, slow addition giving optimum results.[29] True globulin turbidities are not obtained below pH 4.5. Hydrolysis with loss of ammonia precludes heating the ammonium sulfate solution in its preparation. The maximum error is 10 per cent. Results are read visually [30] or by the photometer.[31] The method is also applied to precipitation with phosphotungstic acid.[32] and trichloroacetic acid.[33] Results agree with those with sulfosalicylic acid.

Fractionation with quaternary ammonium salts has been proposed.[34] Serum proteins are also fractionated with lithium sulfate.[35] Total protein is precipitated at pH 6.6 by 15 minutes' heating at 100° with 0.8 saturated magnesium sulfate and 0.05 saturated ammonium sulfate. The colloidal dispersion obtained with mercuric chloride [36] shows greater sensitivity than that with either trichloroacetic acid or sulfosalicylic acid.

Methods for the various fractions of protein are included under the

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26 Carlos A. Sagastume, Danilo Vucetich, and Raul Nico, *Rev. facultad cienc. quím.* **13**, 67-71 (1938).

27 N. I. Joukovsky and Rita Vandervelden, *Ind. chim. belge.* **17**, 251-4 (1952).

28 Stefan Rusznyak, *Biochem. Z.* **133**, 370-2 (1922); A. Koranyi and E. R. Hatz, *Z. anal. Chem.* **97**, 266-70 (1934); Gerhard Schneider, *Z. physiol. Chem.* **283**, 112-26 (1948); B. Levin, V. G. Oberholzer, and T. P. Whitehead, *J. Clin. Path.* **3**, 284-6 (1950).

29 E. J. Fredenburgh and B. P. Hecht, *Am. J. Clin. Path.* **22**, 592-7 (1952).

30 A. Sols, *Rev. españ. fisiol.* **1**, 72-6 (1945).

31 Paul Ujsághy, *Biochem. Z.* **307**, 264-9 (1941).

32 F. Z. Zorilla, *Rev. clin. españ.* **34**, 31-6 (1949).

33 Hilfred N. Bossak, Arthur A. Rosenberg, and Ad Harris, *J. Venereal Disease Inform.* **30**, 100-3 (1949).

34 F. J. Loomeijer, *Nature* **166**, 951-2 (1950).

35 L. Jandrassik and A. Polgár, *Biochem. Z.* **305**, 237-42 (1940).

36 D. M. Kobuledze, *Lab. Prakt.* (USSR) **1939**, No. 2-3, 35-6; Eugenio E. Vonesch, *Anales farm. bioquím.* (Buenos Aires) **11**, 54-62 (1940).

appropriate titles. Proteins, separated as fractions and redissolved, are estimated nephelometrically after controlled addition of trichloroacetic acid.[37] As an indirect method add excess of a solution containing 0.4 per cent of tannin and 0.1 per cent of phenol.[38] After precipitation is complete, read excess tannin in the upper layer.

Reagents—For convenience a series of reagents used in many determinations is listed.

Sulfuric-phosphoric acid mixture. Mix 300 ml. of 85 per cent phosphoric acid with 100 ml. of concentrated sulfuric acid. Put in a tall cylinder, cover, and let stand a week or so for sedimentation of calcium sulfate. To 100 ml. of clear supernatant liquid add 10 ml. of a 6 per cent copper sulfate solution and 100 ml. of water.

Protective colloid solution. Add slowly, stirring vigorously, 10 grams of powdered gum arabic in 190 ml. of water. Add 4 grams of Permutit powder and shake at intervals for 10 minutes. The supernatant liquid should give only a very faint color with Nessler's reagent. Insoluble matter settles out after some hours' standing. Treat the supernatant liquid with one-tenth volume of Nesslers' reagent (page 181), let stand, and decant the clear liquid. Use 3 ml. of this with 15 ml. of Nessler's reagent when the nitrogen content of the sample is high.

Buffer for pH 7. Mix 29.6 ml. of 0.8 per cent carbonate-free sodium hydroxide solution and 50 ml. of 2.72 per cent monopotassium phosphate solution, and dilute to 100 ml.

Neutral sodium sulfate solution Dissolve 650 grams of crystallized sodium sulfate decahydrate in water and dilute to 1 liter. To correct for any slight acidity of the product and for incomplete hydration carry out the following determinations. Dilute 20 ml. of solution to about 50 ml. and titrate with 0.4 per cent sodium hydroxide solution to neutrality to phenolphthalein. Add the calculated amount of sodium hydroxide solution to neutralize the remaining 980 ml. Pipet out 2 ml. of the solution, evaporate to dryness, ignite, and weigh. From this calculate the volume of solution required to make any specified concentration. Store at 35-40° when not in use, to prevent crystallization of the decahydrate.

[37] St. Ruznijak, *Biochem. Z.* **133**, 370-2 (1922); St. Ruznijak and I. Barrat. *Ibid.* **141**, 476-8 (1923); A. A. Klement'eva, *Lab. Prakt.* (USSR) **14**, No. 11, 16-20 (1939).

[38] H. Ravier, *Ann. biol. clin.* (Paris) **8**, 604-6 (1950).

Sample—*Plasma.* For development with phosphotungstic-phosphomolybdic acid, dilute the plasma with 9 volumes of 0.9 per cent sodium chloride solution. Mix 1 ml. with 4 ml. of water and 1 ml. of 20 per cent trichloroacetic acid solution. Centrifuge and thereafter dissolve the precipitate in 0.5 ml. of 10 per cent sodium hydroxide solution by heating in boiling water for one-half hour using an air condenser.

For development with α-naphthol, mix 0.5 ml. of plasma with 10 ml. of 10 per cent trichloroacetic acid and centrifuge after 10 minutes. Decant the supernatant layer for determination of nonprotein nitrogen by the Kjeldahl method. Suspend the protein in 5 ml. of 5 per cent trichloroacetic acid, centrifuge, and decant to the previous decantate. Dissolve the residue in 10 ml. of 10 per cent sodium hydroxide solution for estimation in an aliquot.

For turbidimetric development with sulfosalicylic acid,[39] dilute 0.2 ml. of plasma to 20 ml. with 0.85 per cent salt solution. As another method prepare a mixture of 2 volumes of saturated aqueous ammonium sulfate and 1 volume of 1:30 hydrochloric acid. Add 3 ml. of this to 1 ml. of centrifuged sample. After 15 minutes read nephelometrically.

Serum. Dilute 0.25 ml. of serum to 5 ml. with 0.9 per cent salt solution. Dilute to 10 ml. with 10 per cent trichloroacetic acid solution. After 5 minutes centrifuge and decant the clear upper layer. Mix the precipitate with 10 ml. of 5 per cent trichloroacetic acid solution, let stand for 5 minutes, and centrifuge. Add 5 ml. of 7.5 per cent sodium hydroxide solution to the residue in the tube. When the precipitate has dissolved, dilute to 9 ml. and develop by the biuret reaction. A suggested standard for the purpose is fibrin or fibrinogen.[40]

For development with phosphotungstic-phosphomolybdic acid, dilute 1 ml. of serum to 10 ml. with 0.9 per cent sodium chloride solution. Mix 0.5 ml. of the diluted serum with about 2 ml. of water. Add 1 ml. of 10 per cent sodium hydroxide solution and heat in boiling water for 10 minutes. Cool at once for use as sample and keep in ice water during the period of color development. Alternatively,[41] mix 0.2 ml. of serum, 9 ml. of water, and 0.2 ml. of 20 per cent sodium hydroxide solution. A method by nitric acid is given near the end of the procedures.

Spinal fluid. Dilute 2 ml. of spinal fluid to 10 ml. Add 2 ml. of

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[39] Colin A. Mawson, *Biochem. J.* **36**, 273-80 (1942).

[40] Augustin D. Marenzi and Carlos Jorge Gomez, *Pubs. centro invest. tisiol.* (Buenos Aires) **15**, 245-52 (1951).

[41] Gerhard Fuhrmann, *Artzl. Wochschr.* **1**, 363-4 (1946).

20 per cent trichloroacetic acid and mix. Heat in boiling water for 1 minute. Let cool and add 6 ml. of absolute methanol. Mix and centrifuge. Decant the supernatant liquid and drain for 5 minutes on filter paper. Washing is not required. Add 2 ml. of the sulfuric-phosphoric acid mixture. Heat in an inclined position over a micro burner until the precipitate is dissolved. Evaporate the water and, when the solution begins to turn brown or when dense white fumes appear, place the tube upright. Cover with a watch glass and continue to heat until colorless. Cool until the fumes subside, tilt, and add about 5 ml. of water, drop by drop. If crystals form, break them up with a stirring rod and rinse the rod. Let cool and dilute to 10 ml. Centrifuge if necessary and transfer 5 ml. of the clear liquid. Add 1 ml. of 10 per cent Rochelle salt solution and dilute to 10 ml. for development with Nessler's reagent.

For development by the biuret reaction, for 10-500 mg. of protein per 100 ml., use without dilution.

For development with phosphotungstic-phosphomolybdic acid, mix 2 ml. of spinal fluid with 1 ml. of 10 per cent sodium tungstate solution and 1 ml. of 1:54 sulfuric acid. Centrifuge after 30 minutes, pour off the supernatant liquid, and add 2-3 drops of 1 per cent sodium hydroxide solution to dissolve the precipitate.

Alternatively,[42] mix 2 ml. of spinal fluid with 3 ml. of water and 1 ml. of 20 per cent trichloroacetic acid. After a few minutes for flocculation, centrifuge, and discard the decantate. Heat the precipitate with 0.25 ml. of 10 per cent sodium hydroxide solution in boiling water for 10 minutes. Add 3.75 ml. of water and develop with phosphotungstic-phosphomolybdate reagent.

For development as molybdenum blue, to 1 ml. of spinal fluid add 1 ml. of 1 per cent phosphomolybdic acid solution in 1:35 sulfuric acid. Mix and let stand for 15 minutes. Centrifuge and decant the supernatant liquid to waste. Suspend the precipitate in 2 ml. of water. Centrifuge and decant as before. The washing insures complete removal of unprecipitated phosphomolybdic acid.

For normal concentrations, dilute 0.6 ml. to 1 ml. for development with sulfosalicylic acid. A method by mercuric nitrate is given near the end of the procedures.

Urine. Dilute an appropriate volume of urine, according to protein

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[42] George W. Johnson and R. B. Gibson, *Am. J. Clin. Path.* **8**, Tech. Suppl. 1, 22-31 (1938).

content, to 10 ml., add 1 ml. of 50 per cent trichloroacetic acid solution, and mix well. After standing for 10 minutes at room temperature, place in a water bath at 50° to flocculate. Centrifuge and decant. Wash four times with 4 per cent trichloroacetic acid, mix well each time, heat at 50°, and centrifuge. After the final washing dissolve the precipitate in 2 drops of 10 per cent sodium hydroxide solution and dilute to 10 ml.

Treat as for total nitrogen in blood (page 183). The high silicate content will usually require centrifuging before comparison.

For development by the biuret reaction, adjust the acidity or alkalinity of a sample to approximate neutrality to litmus. About pH 7.4 is best. Take a sample of 1 ml., if about 1 per cent of total protein is present, or a modified volume for different protein content. For low protein content use 4 ml. Treat as for total protein in serum (page 177), starting at "Dilute to 10 ml. with 10 per cent trichloroacetic acid solution."

For development with phosphotungstic-phosphomolybdic acid dilute 1-5 ml. of filtered urine to 10 ml. with water. Add 2 ml. of 10 per cent sodium tungstate solution and 2 ml. of 1:54 sulfuric acid. Mix well. If the urine was alkaline, the supernatant liquid may be milky. In that case add a few more drops of the sulfuric acid. Mix, centrifuge, and decant. Add 1 ml. of 10 per cent sodium tungstate solution to the precipitate and stir until dissolved. Dilute with 10 ml. of water and add 1 ml. of 1:54 sulfuric acid. Mix, centrifuge, and decant. Repeat the process of solution and precipitation again to remove nonproteins which react with the reagent. If the volume of precipitate is 0.4 ml. or more, suspend in 50 ml. of water and dissolve with 2-3 drops of 1 per cent sodium hydroxide solution. If the volume is much less than 0.4 ml., discard and repeat the process with a larger volume of urine. If the volume of precipitate exceeded 0.5 ml., dilute to 10 ml. and take an aliquot for the procedure.

For development with α-naphthol, mix 5 ml. with 2 ml. of 50 per cent trichloroacetic acid. Centrifuge after 10 minutes and discard by decantation. Resuspend the protein in 5 ml. of 5 per cent trichloroacetic acid, centrifuge, and decant. Dissolve the protein in 5 ml. of 10 per cent sodium hydroxide for estimation.

Proteins from urine are determined nephelometrically with ammonium sulfate. Neutralize a filtered sample. As a sample mix 9.5 ml. of saturated ammonium sulfate solution and 0.5 ml. of urine. This pre-

cipitates all the proteins. Analogous methods for globulin, fibrinogen and euglobulin, and fibrinogen are given under the appropriate topics. Read nephelometrically without separation from the mother liquor. A method by mercuric nitrate is given near the end of the procedures.

Ascitic fluid. Treat as for urine and develop with α-naphthol.

Gastric juice.[43] Make gastric juice homogeneous by 1-2 drops of 33 per cent sodium hydroxide solution. Develop a portion with sulfosalicylic acid.

Milk. For nephelometric development with sulfosalicylic acid, mix 5 ml. of milk with 200 ml. of water and 10 ml. of 0.4 per cent sodium hydroxide solution. Dilute to 250 ml. and shake. Shake 10 ml. with 2 ml. of ether to extract the fat. Let stand until the layers separate and withdraw 5 ml. of the aqueous layer without ether. Dilute to 50 ml.

Animal products.[44] Heat 2 ml. of solution at 75° for 4 minutes. Add 2 ml. of 0.4 per cent sodium hydroxide solution and heat for 2 minutes at 75°. To precipitate the protein add 6 drops of 25 per cent acetic acid and 2 ml. of saturated magnesium sulfate solution. Heat in boiling water for 3 minutes to coagulate. Filter and wash the precipitate with hot water. Dissolve the precipitate in 15 ml. of 5 per cent sodium hydroxide and develop an aliquot by the biuret reagent.

Plankton.[45] Treat a salt-free sample dried at 95° with 8 per cent sodium hydroxide solution at room temperature for 24 hours. Dilute to 10 times the volume and let stand for 2 hours. Filter and develop an aliquot by the biuret reaction. For this the concentration of sodium hydroxide is built up to 4 per cent by mixing with an equal volume of 7.6 per cent sodium hydroxide solution. Alternatively use a special reagent made by mixing 6 ml. of 2 per cent copper sulfate solution and 50 ml. of ethylene glycol.

Vegetable flours. Grind 0.5 gram of flour and 3 grams of sand with 25 ml. of 2 per cent sodium hydroxide solution in 50 per cent ethanol for 1 hour. Centrifuge and develop an aliquot with the biuret reagent.

Grains and seeds. Grind a 0.5-gram sample with 5 grams of sand. Extract for 1 hour with 20 ml. of 0.2 per cent sodium hydroxide solution in 50 per cent ethanol. Filter and add 1.5 ml. of 10 per cent sodium hydroxide solution to 10 ml. of filtrate. Develop with salicylic acid.

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43 Friedrich Baltzer, *Arch. Verdauungs Krankh.* 62, 113-56 (1937).
44 L. M. Kul'berg and P. A. Soĭfer, *Biokhimiya* 12, 1-6 (1947).
45 Johannes Krey, *Kieler Meeresforschungen* 8, No. 1, 16-29 (1951).

Procedure—*By Nessler's reagent.* As reagent, dissolve 100 grams of mercuric iodide and 70 grams of potassium iodide in about 400 ml. of water. Dissolve 100 grams of sodium hydroxide in about 500 ml. of water. Cool this solution of alkali thoroughly and add to the previous mixture with constant shaking. Dilute to 1 liter and let stand. A small amount of brownish to red precipitate usually settles to leave a clear supernatant liquid. Pour off the clear layer and use. Add 15 ml. of Nessler's reagent to the sample and dilute to 50 ml. or 100 ml. Mix and read at 410 mμ.

Nephelometric Nessler's reagent. As reagent mix 80 grams of sodium chloride and 130 ml. of water. Add 100 ml. of a cold saturated solution of mercuric chloride. Shake well and, when the precipitate has dissolved, add slowly with shaking 70 ml. of a saturated lithium carbonate solution. Be sure that no mercuric oxide is deposited on the walls of the flask. The solution will show a cloud due to the ammonia in the reagents. Add 5 grams of finely divided talc and shake. Filter to obtain a clear solution. This will keep several weeks if well stoppered. To the neutralized sample add 0.1 ml. of a fresh 1 per cent starch solution and disperse well. Add half the volume of the reagent and read nephelometrically.

By the biuret reaction. Add 0.2 ml. of 20 per cent solution of copper sulfate pentahydrate to a prepared 5-ml. sample in 4 per cent sodium hydroxide solution. Mix and shake for 10 seconds. After 5 minutes for precipitation of cupric hydroxide, centrifuge and decant the upper layer for reading at 530 mμ against a reagent blank between 30 and 90 minutes after adding the reagent.

By phosphotungstic-phosphomolybdic acid. Solutions. To 0.2 ml. of sample containing 0.005-0.1 mg. of protein add 1 ml. of a fresh 1:1 mixture of 2 per cent sodium carbonate in 0.4 per cent sodium hydroxide solution and 0.5 per cent solution of cupric sulfate pentahydrate in 1 per cent sodium tartrate solution. Titrate phenol reagent (Vol. III, page 116) to a phenolphthalein end point and on the basis of the titration dilute so that the acidity is 1 *N*. Add 0.1 ml. of this after the previous mixture has stood for 10 minutes and mix at once. Read after 30 minutes at 750 mμ for dilute samples or 500 mμ for more concentrated ones.

Protein precipitates. To 0.005-0.1 mg. of protein add 0.1 ml. of 4 per cent sodium hydroxide solution. After this has dissolved add 1 ml. of a fresh 1:1 mixture of 2 per cent sodium carbonate solution

and 0.5 per cent solution of cupric sulfate pentahydrate in 1 per cent sodium tartrate solution. Complete as for the solutions from "Titrate phenol reagent . . .."

As molybdenum blue. Add to the precipitate of protein phosphomolybdate 1 ml. of 1:180 sulfuric acid and 1 ml. of 2 per cent hydroquinone solution, and shake. Add 1 ml. of a solution containing 15 per cent of sodium carbonate and 3 per cent of sodium sulfite. Read promptly at 610 mμ against a reagent blank.

By α-naphthol. To 1 ml. of protein solution in 10 per cent sodium hydroxide solution add 5 ml. of water and 1 ml. of 0.1 per cent solution of α-naphthol in ethanol. Mix and after 5 minutes add 1 ml. of 0.46 per cent sodium hypochlorite solution. Exactly 1 minute later add 2 ml. of 20 per cent urea solution. After 5 minutes read at 540 mμ against a reagent blank. An arginine standard may be used and multiplied by 19.2 for protein.

Hydrolyzates as copper salts. As buffer for pH 9.1-9.2 dissolve 6 per cent of sodium chloride in filtered 4.03 per cent solution of anhydrous sodium tetraborate. To 5 ml. of buffer solution add 5 ml. of aqueous solution of the test substance. Add 0.1 ml. of 0.852 per cent solution of cupric chloride, mix, and let stand for 10 minutes. Centrifuge and read at 230 mμ against an alanine check solution. Read in terms of alanine and interpret in terms of the values in Table 12.

By sulfosalicylic acid. Transfer a 1-ml. sample to a tube of about 4 ml. capacity. Add 1 ml. of 5 per cent sulfosalicylic acid solution. Mix by inversion, but not by violent shaking. Let stand for a definite period from 5 minutes to 5 hours and read the turbidity at 700 mμ or for low concentrations read at 430 mμ. Subtract a reagent blank.

Turbidimetric by ammonium sulfate. Shake the tube, let stand for 20 minutes, and read at 500 mμ.

Turbidimetric by trichloroacetic acid. Add 9.5 ml. of a 30 per cent solution of trichloroacetic acid to 0.5 ml. of sample. Mix well and read at 530 mμ after 20 minutes.

By nitric acid.[46] *Serum.* Mix 1 ml. of the sample with 1 ml. of 1:1 nitric acid and heat exactly 25 minutes in boiling water. Cool and add 1.5 ml. of concentrated ammonium hydroxide. Read after 15 minutes at 430 mμ against a reagent blank.

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[46] Michael A. Macheboeuf and Pierre Rebeyrotte, *Compt. rend. soc. biol.* **141**, 266-7 (1947); Michael A. Macheboeuf, Paulette Lacaille, and Pierre Rebeyrotte, *Bull. soc. chim. biol.* **29**, 402-11 (1947).

By mercuric chloride. Spinal fluid. Dilute 0.1 ml. to 1.5 ml. with water. Add 0.5 ml. of reagent containing 2 per cent of mercuric chloride, 2 per cent of sodium chloride, and 4 per cent of succinic acid. Incubate at 37° for 30 minutes and read at 420 mμ against a reagent blank.

TABLE 12.—ALANINE EQUIVALENCE OF COPPER SALTS OF 0.0003 M SOLUTIONS OF AMINO ACIDS MEASURED AT 230 Mμ

Amino Acid	*Wave Lengths of Maximum Absorbancy*	*Alanine Equivalence*	
		At 230 mμ	*At Wave Length of Maximum Absorbancy*
	mμ	*per cent*	*per cent*
Alanine		100.0	
Arginine	229-230	103.3	
Aspartic acid	229-232	96.9	
Cystine 0.00015 *M*	232-236	80.9	83.7
Glutamic acid	229-233	103.8	
Glycine	226-227	100.2	102.7
Histidine	225-227	83.5	87.1
Hydroxyproline	237-239	91.1	98.1
Isoleucine	230-232	102.7	
Leucine	228-233	103.9	
Lysine	230-232	98.9	
Methionine	229-230	102.6	
Phenylalanine	230-235	91.1	
Proline	239-240	83.5	90.4
Threonine	230-233	100.7	
Tryptophan		144.1	
Tyrosine	232-233	94.7	97.7
Valine	230-231	101.5	

Urine. As reagent dissolve 2 per cent of mercuric chloride, 0.4 per cent of sodium chloride, and 0.4 per cent of tartaric acid. Add 0.5 ml. to 5 ml. of urine. Read at 420 mμ against a reagent blank.

TOTAL NITROGEN

Total nitrogen is a sum of protein forms of nitrogen plus nonprotein nitrogen which in turn includes free ammonia.

Sample—*Blood.* Digest a sample containing 2-5 mg. of nitrogen, with 2 ml. of a mixture containing 70 ml. of concentrated sulfuric acid, 50 ml. of water, 20 ml. of 20 per cent perchloric acid, 15 grams of anhydrous sodium sulfate, and 1 gram of copper sulfate. Heat for at

least 2 minutes after the solution is colorless. When cool, add 2.5 ml. of the protective colloid solution described under reagents for total protein (page 176). Dilute to nearly 35 ml. for development with Nessler's reagent.

Plasma. Dilute 1 ml. of plasma to 100 ml. Continue as for total nitrogen in blood, starting at "Digest a sample containing 2-5 mg. of nitrogen . . . ."

Alternatively,[47] dilute 20 cu. mm. of sample with 0.5 ml. of water. Add 0.25 ml. of concentrated sulfuric acid and heat to fumes of sulfur trioxide. Boil for 3 minutes and cool. Add 0.5 ml. of saturated potassium persulfate solution. Heat to sulfur trioxide fumes and cool. Dilute to 25 ml. and develop a 5-ml. aliquot by Nessler's reagent.

Urine. Follow the method for total nitrogen in blood with a diluted urine, starting at "Digest a sample containing 2-5 mg. of nitrogen . . . ."

Milk. Use a diluted sample and follow the method for total nitrogen in blood, starting with "Digest a sample containing 2-5 mg. of nitrogen . . . ."

Grain.[48] Digest 0.5 gram with 5 ml. of concentrated sulfuric acid and 2 ml. of 30 per cent hydrogen peroxide for 5 minutes on a sand bath. Cool, add 3 ml. more of hydrogen peroxide, and digest for 30 minutes. Cool, dilute to a known volume, and determine by Nessler reagent.

Foodstuffs, etc. Heat 0.01 to 0.04 gram of well-sampled rye flour, wheat flour, bread, beef, dried fish, milk, cheese, tobacco, or leather with 2 ml. of concentrated sulfuric acid and a few drops of 30 per cent hydrogen peroxide. Heat very carefully until the reaction starts as it is likely to be vigorous. Allow to cool. Add a few more drops of 30 per cent hydrogen peroxide and heat. Repeat until the contents of the tube are substantially colorless. This will usually require 0.3-0.5 ml. of hydrogen peroxide. If necessary oxidize several individual samples and combine the results of acid digestion, rather than increasing the amount of sample over 0.04 gram.

Neutralize the sample with 5 per cent carbonate-free sodium hydroxide solution until it is no longer acid to methyl orange but is not alkaline to phenolphthalein. Dilute to nearly 35 ml. for development with Nessler's reagent.

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[47] W. W. Walther, *Lancet* **1941, II,** 337-8.

[48] Fritz Weir, *Landw. Jahrb.* **84,** 1-25 (1937).

Gelatine in muscle.[49] Autoclave 0.1-0.2 gram of desiccated muscle with 4 ml. of water at 15-20 lbs. for 2 hours. Cool, centrifuge, and decant. Wash the residue with 3 two-ml. portions of water. Acidify the aqueous solution containing all of the gelatine with 5 ml. of 5 per cent tannic acid solution and store at 0° for 1 hour. Centrifuge, decant, and dissolve the residue in 8 ml. of 1 per cent sodium hydroxide solution. Neutralize with 1:17 sulfuric acid and digest with 2 ml. of 1:1 sulfuric acid-30 per cent hydrogen peroxide. Dilute the clear solution to 100 ml. with water and Nesslerize an aliquot.

Petroleum Oils.[50] To about 1 gram of sample in a Kjeldahl flask add 0.1 gram of salicylic acid and about 0.2 gram of a 5:2:1:1 mixture of potassium sulfate, mercury, cupric sulfate, and selenium. Add about 8 ml. of concentrated sulfuric acid, and let stand for about 10 minutes. Heat under a hood until clear, usually 30-120 minutes. More acid may be required. Continue to digest for 1 hour, cool, and dilute with about 10 ml. of water.

Connect up for steam distillation with steam from water slightly acidified with sulfuric acid. Cautiously neutralize with 50 per cent sodium hydroxide from a dropping funnel mounted as part of the apparatus, and add 1 ml. excess. Steam-distil, collecting 50 ml. for development with Nessler's reagent.

Procedure—*By Nessler's reagent.* Determine as described (page 181) under total protein.

Free Ammonia

Determination of free ammonia as present in solution with proteins is, of course, not a part of either the total proteins or any fraction. It is often necessary for subtraction from the total nitrogen to give total protein content.

Sample—*Urine.* Wash 2 grams of Permutit by decantation, twice with ammonia-free water, once with 2 per cent acetic acid, and finally twice more with ammonia-free water. After this add 0.5 ml. of urine and 10-15 ml. of water. Shake for 5 minutes. Wash down the sides and

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49 Howard C. Spencer, Sergius Morgulis, and Violet M. Wilder, *J. Biol. Chem.* **120**, 257-66 (1937).

50 Cecil H. Hale, Margie N. Hale, and William H. Jones, *Anal. Chem.* 21, 1549-51 (1949).

decant. Wash twice with ammonia-free water and decant. Add about 150 ml. of ammonia-free water. Cool and add 10 ml. of 10 per cent sodium hyroxide solution. Dilute to 200 ml. and use an aliquot for development with Nessler's reagent by the procedure under total protein (page 181).

Globulin

Globulins are precipitated by half-saturated ammonium sulfate[51] solution or by 23 per cent sodium sulfate solution. The resulting solid fraction is dissolved and determined by any of the methods for total protein. Also, the ninhydrin reaction is applied to the solution.[52]

Sample—*Serum.* Dilute 0.5 ml. of serum to 10 ml. with 27.79 per cent ammonium sulfate solution. Heat in boiling water for 30 minutes. When precipitation is complete, centrifuge and decant the upper layer for separation and determination of albumin (page 188). Dissolve the precipitate and complete as for total protein by the biuret reaction (page 181).

For development with phosphotungstic-phosphomolybdic acid, mix 0.5 ml. of serum with 9.5 ml. of 22.5 per cent anhydrous sodium sulfate solution and set aside at 37° for 2 hours. Filter through paper, repeating if the filtrate is not clear. Reserve the filtrate for determination of albumin. Wash the globulin residue from the test tube onto the filter with two 3-ml. portions of 22.5 per cent sodium sulfate solution. Wash the precipitate on the filter with two 3-ml. portions of the 22.5 per cent sodium sulfate solution. Dissolve the globulin with 0.04 per cent sodium hydroxide solution. Continue to wash until made up to about 25 ml. Add 2 ml. of 20 per cent sodium hydroxide solution and dilute to 50 ml. Use 10 ml. for development by phosphotungstic-phosphomolybdic acid (page 181).

Plasma. Mix 0.5 ml. of plasma with 10 ml. of 22 per cent sodium sulfate solution and incubate at 37° for 3 hours. Add 3 ml. of ether, shake vigorously, and centrifuge. Protein separates at the interface. Decant and resuspend the cake in 10 ml. of 22 per cent sodium sulfate solution. Add 3 ml. of ether, shake, and centrifuge. Decant and dissolve the globulin in 10 ml. of 10 per cent sodium hydroxide solution

[51] George R. Kingsley, *J. Biol. Chem.* **131**, 197-200 (1939); Andrew F. Braff and James M. Cobb, *U. S. Naval Med. Bull.* **49**, 753-6 (1949).

[52] Abraham Seifer and Michael C. Zymaris, *J. Clin. Invest.* **31**, 1-11 (1952).

by gentle warming to drive off the ether. Develop an aliquot by α-naphthol (page 182).

Spinal fluid. To 5 ml. of spinal fluid in a tube add 5 ml. of saturated ammonium sulfate solution. Place the tube in a water bath at 56° until maximum precipitation is obtained—about 30 minutes. Centrifuge about 10 minutes. Pour off the supernatant liquid and drain thoroughly.

Add 1 ml. of 10 per cent sodium tungstate solution and mix until the precipitated globulin dissolves. Add 4 ml. of water and 1 ml. of 1:54 sulfuric acid. Globulin is precipitated again. Centrifuge and discard the supernatant liquid. Add 2 drops of 1 per cent sodium hydroxide solution and dilute to an appropriate volume for development by phosphotungstic-phosphomolybdic acid (page 181).

For determination by nitric acid,[53] mix 2 ml. with 2 ml. of saturated ammonium sulfate solution. Centrifuge after 2 hours to separate the globulin fraction and decant the upper layer containing albumins for separate determination. Use the precipitate in the procedure.

Urine. Separate as described for the first method for globulin in serum (page 186) and determine by the biuret reaction (page 181). As an alternative take an amount of urine such that it will yield 8-20 mg. of globulins. Heat to 38°. Add an equal volume of 44 per cent sodium sulfate solution and maintain at 38° for 3 hours. Centrifuge and decant. Dissolve the precipitate and complete as usual for protein fractions by the Kjeldahl reaction and determination by Nessler's reagent.

For determination by α-naphthol, mix 10 ml. of urine with 10 ml. of 44 per cent sodium sulfate and incubate at 37° for 3 hours. Centrifuge and decant to waste. Resuspend the precipitate in 10 ml. of 22 per cent sodium sulfate solution. Add 3 ml. of ether, shake vigorously, and centrifuge. Decant to waste and dissolve the precipitate in 10 ml. of 10 per cent sodium hydroxide solution for determination (page 182).

For reading as turbidity (page 182) without separation from the mother liquor mix 5 ml. of saturated ammonium sulfate solution, 4.5 ml. of water, and 0.5 ml. of urine.

Ascitic fluid. Follow the technic for urine.

Procedure—*By nitric acid.* Treat the globulin precipitate with 0.5 ml. of 1:2 nitric acid and heat in boiling water for exactly 5 minutes.

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[53] Wilhelm Ederle, *Deut. med. Wochschr.* 74, 1411-12 (1949).

Cool and add 0.5 ml. of 33 per cent sodium hydroxide solution. Read at 430 mμ against a reagent blank.

Albumin

Globulin is precipitated from solution by half-saturated ammonium sulfate. The resulting liquid fraction contains the albumin which is determined by any of the methods for protein. An appropriate technic is the trichloroacetic acid precipitation after globulin is removed.[54]

Sample—*Serum.* The sample is an upper layer decanted from globulins (page 186) or a filtrate similarly obtained. Add 5 ml. of 10 per cent trichloroacetic acid to 5 ml. of filtrate and let stand for 5 minutes. Centrifuge and decant. Mix the precipitated albumin with 10 ml. of 5 per cent trichloroacetic acid, centrifuge after 5 minutes, and decant. Dissolve the precipitate in 5 ml. of 4 per cent sodium hydroxide solution for development by the biuret reaction (page 181).

Plasma. Mix 1 ml. of plasma with 2 ml. of buffer for pH 7 and sufficient neutral sodium sulfate solution so that the final concentration is 22.05 per cent of sodium sulfate. Dilute to 50 ml., mix, and let stand at room temperature for 1 hour. Filter through a fine grade of paper until clear. Proceed with 10 ml. of filtrate, digesting as for nonprotein nitrogen in blood (page 191) and developing with Nessler's reagent (page 181).

For the biuret reaction, add 1 ml. of plasma to 24 ml. of 28 per cent sodium sulfate. Mix and filter, discarding the first 5 ml. of filtrate. Develop with the biuret reagent (page 181) to get albumin. Determine globulin by difference from the total protein.

For development by phosphotungstic-phosphomolybdic acid, add 1 ml. of plasma to 9 ml. of a saturated solution of magnesium sulfate and 0.3 gram of anhydrous magnesium sulfate. Mix and let stand for 30 minutes. Filter and mix 1 ml. of filtrate with about 12 ml. of water, 1 ml. of a 10 per cent sodium tungstate solution, and 1 ml. of 1:54 sulfuric acid. Stir and centrifuge. Decant as completely as possible and add to the precipitate 10 ml. of water and 2 drops of 20 per cent sodium carbonate solution. Stir until the precipitate has dissolved and dilute to 25 ml. for use of an aliquot (page 181).

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[54] H. Surmont and R. Provino, *Bull. soc. chim. biol.* **10**, 413-14 (1928); Halvor N. Christensen, *J. Lab. Clin. Med.* 31, 916-17 (1946); A. Jond and Ta[illegible], *Ann. biol. clin.* (Paris) **8**, 498-500 (1950).

Urine. Weigh 11.02 grams of neutral anhydrous sodium sulfate. Add 5 ml. of buffer solution for pH 7 (Vol. I, page 176) and 10-40 ml. of urine according to protein concentration. Dilute nearly to 50 ml. with water. Put in a water bath at 45° and shake frequently until the salt is dissolved. Let cool to room temperature and dilute to 50 ml. After one hour filter through fine filter paper, refiltering as necessary until a clear filtrate is obtained. Use 5 or 10 ml. of filtrate, treating as for total nitrogen in blood (page 183). Correct for a separate determination of nonprotein nitrogen.

For development by the biuret reaction, adjust the acidity or alkalinity of a sample to approximate neutrality to litmus. About pH 7.4 is best. Take a sample of 3 ml. if about 1 per cent of total proteins is present or a modified volume for different protein contents. For low protein content use 12 ml. Mix with an equal volume of saturated ammonium sulfate solution to precipitate globulin. Filter and pipet out 5 ml. of filtrate for further treatment. Dilute this to 10 ml. with 10 per cent trichloroacetic acid and treat as for total protein in serum (page 177), starting at "After 5 minutes centrifuge . . .."

Gastric juice. Dilute 1:10 or 1:20 so as to bring the concentration of albumin to 0.03-0.1 per cent. Develop turbidimetrically by trichloroacetic acid (page 182).

Spinal fluid. Globulin was separated and the balance saved. Precipitate the albumins with 2 ml. of 20 per cent trichloroacetic acid. Centrifuge after 1 hour and decant the upper layer to waste. Develop the precipitate by nitric acid (page 187).

Fibrinogen

Fibrinogen is precipitated by calcium chloride or sodium sulfite[55] and thereafter determined by the usual methods for protein fractions. Thus it is determined by reaction with phosphotungstic-phosphomolybdic acid, due largely to the tyrosine which it contains, to give the usual blue color.[56]

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55 Leonel Fierro del Rio, Delfina Arrieta Aupart, and Florencia Cano Vega, *Rev. inst. salubridad y enfermedad. trop.* (Mex.) **9**, 87-94 (1948); Blanca A. Mendioroz, *Arch. uraguayos med., cinq. y. especial.* **35**, 271-4 (1949).

56 Hsien Wu, *J. Biol. Chem.* **51**, 33-9 (1922); Hsien Wu and Schmori W. Ling, *Chinese J. Physiol.* **1**, 161-8 (1927); A. T. Cameron, J. S. Guthrie and F. D. White, *Can. Med. Assoc. J.* **35**, 32-7 (1936).

Samples—*Serum.* Mix 2 ml. of oxalated plasma with 60 ml. of 0.85 per cent sodium chloride solution. Add 2 ml. of a 5 per cent solution of calcium chloride hexahydrate and mix. Centrifuge and decant from the clotted material. Wash thrice with 0.85 per cent sodium chloride solution. Dissolve the precipitate in 1 ml. of 30 per cent sodium hydroxide solution and dilute to 9 ml. with water for determination by the biuret reaction (page 181).

For turbidimetric reading, precipitate 2 ml. of sample with 1.08 ml. of saturated ammonium sulfate reagent. Do not separate from the mother liquid for reading (page 182).

Plasma. Dilute 0.5 ml. of plasma to 10 ml. with 12.5 per cent sodium sulfite solution. Incubate at 37° for 10 minutes and centrifuge. Decant to waste and suspend the precipitate in 7 ml. of 12.5 per cent sodium sulfite solution. Centrifuge and decant. Dissolve the precipitate in 10 ml. of 3 per cent sodium hydroxide solution for development by the biuret reaction (page 181).

Alternatively,[57] mix 1 ml. of plasma with 1.25 ml. of 5 per cent calcium chloride solution and dilute to 50 ml. with 0.7 per cent sodium chloride solution. Transfer to a centrifuge tube and incubate until fibrin separates. Centrifuge and decant. Wash the precipitate with 0.7 per cent sodium chloride solution until the washings show a negative test for calcium. Wash the precipitate successively with 3 ml. of ethanol, 3 ml. of 1:1 ethanol-ether, and 3 ml. of ether. Dry and dissolve in 5 ml. of 4 per cent sodium hydroxide solution for the biuret reaction (page 181).

For development with phosphotungstic-phosphomolybdic acid, to 1 ml. of plasma from blood containing 0.2 to 0.6 per cent of potassium oxalate, add 28 ml. of an 0.8 per cent sodium chloride solution and 1 ml. of 2.5 per cent calcium chloride solution. Mix and let stand 20 minutes. Break up the jelly by shaking slightly and transfer to a dry filter. While filtering, insert a glass rod with a pointed end into the jelly and whirl gently. The fibrin should stick to the rod. If any fibrin fails to stick, pick it up with the tip of the rod. Remove the fibrin from the rod and press between dry filter paper to remove the adhering liquid. Add 4 ml. of a 1 per cent solution of sodium hydroxide. Heat in boiling water and stir with a glass rod until the fibrin has completely dissolved, leaving the calcium oxalate in suspension. Add 10 ml. of water, mix, and centrifuge. Cool the supernatant liquid as the sample.

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[57] Heinz Esser and F. Heinzler, *Klin. Wochschr.* 30, 991-3 (1952).

Alternatively,[58] mix 0.5 ml. of crushed Pyrex glass of particle size under 0.5 mm., 10 ml. of 0.85 per cent sodium chloride solution, 0.05 ml. of thrombin containing 1000 units per ml., and 0.5 ml. of citrated or oxalated plasma. Agitate and after 10 minutes centrifuge. The fibrin is largely deposited on the powdered glass. Wash the deposit twice with 10 ml. of 0.85 per cent sodium chloride solution. Dissolve in 10 ml. of 10 per cent sodium hydroxide solution by heating in boiling water for 10 minutes. Develop with phosphotungstic-phosphomolybdic acid.

Procedure—*By phosphotungstic-phosphomolybdic acid.* Mix 1 ml. of sample solution, 7 ml. of water, and 3 ml. of 20 per cent sodium carbonate solution. Add 1 ml. of reagent (Vol. III, page 116) and read the color at 680 mμ after 15 minutes. Subtract a reagent blank. Interpret in terms of a curve developed with tyrosine and multiply the result by 11.7 to convert tyrosine to fibrinogen.

Nonprotein Nitrogen

The remaining nitrogen after removal of all the protein is the nonprotein nitrogen. In some samples it would be the ammonia nitrogen.

Samples—*Blood.* Treat a sample of protein-free blood filtrate corresponding to 0.3-1 mg. of nitrogen with 1 ml. of 1:1 sulfuric acid. Heat until the tube becomes filled with dense white fumes. Let cool for 15-30 seconds. Add 5 drops of 30 per cent hydrogen peroxide and heat again until white fumes appear. If the solution remains colorless, boil gently for 2 to 5 minutes. If it becomes colored after heating and swirling, add more peroxide and again heat. After complete digestion and cooling, dilute to about 75 ml. for development by Nessler's reagent (page 181).

Alternatively,[59] mix 0.1 ml. of blood with 4 ml. of a 2 per cent ammonium molybdate solution containing 1.3 per cent of potassium sulfate. After 10 minutes add 1 ml. of 1:280 sulfuric acid. Mix well and centrifuge. Digest 4 ml. of the clear supernatant layer for development with Nessler's reagent.

Serum. Add 11 ml. of 2.5 per cent trichloroacetic acid to 1 ml. of serum. Shake vigorously and filter. To 8 ml. of clear filtrate add 1 ml. of a mixture of equal volumes of concentrated sulfuric acid and 0.4 per

[58] Oscar D. Ratnoff and Calvin Menzie, *J. Lab. Clin. Med.* **37**, 316-20 (1951).
[59] Wilhelm Ohlsson, *Skand. Arch. Physiol.* **75**, 207-14 (1937).

cent perchloric acid. Digest until destruction of organic matter is complete. Cool and add about 25 ml. of water. Cool and develop with Nessler's reagent.

Urine. Deproteinize according to the usual technic by phosphotungstic acid. Complete according to the method for total nitrogen in blood (page 183), starting at "Digest a sample containing 2-5 mg. of nitrogen . . .."

Plasma. A sample was separated (page 177) from the total protein for use in this determination.

Liver. Slice the liver very thin. Freeze a sample of about 20 grams with solid carbon dioxide. Rub up the frozen sections quickly with quartz sand in a precooled mortar before they melt. Add 40 ml. of water to produce a thin slurry. Determine the specific volume of the frozen liver by transferring another known weight to a volumetric flask and diluting to volume by adding water from a buret. The volume of the thin slurry is then to be 40 ml. plus the volume of the sample. Deproteinize with phosphotungstic acid and filter. Determine residual nitrogen in the filtrate according to the method for total nitrogen in blood (page 183), starting at "Digest a sample containing 2-5 mg. of nitrogen . . . ."

Albumoses

After separation of other interfering substances, albumoses are determined by a modified Millon's reagent.[60]

Sample—*Urine.* Acidify 100 ml. of urine with acetic acid, filter if necessary, and evaporate to dryness. Take up the residue in water, cool, dilute to 25 ml., and filter. Acidify the filtrate with 3 drops of glacial acetic acid, add 3 volumes of ethanol to precipitate the albumoses, and filter after 1 hour. Wash the filter twice with ethanol and discard the filtrate. Air-dry the paper and then boil with 10 ml. of water. Filter to get a clear-colored albumin solution. If turbidity develops, saturate with solid ammonium sulfate, filter, and take up the precipitate in water.

Procedure—Use that for typrosine by mercuric sulfate and nitrite (page 127).

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[60] M. Wiese, *Acta Med. Scand.* 109, 312-20 (1941).

Polypeptides. Tyrosine Index

Polypeptides are intermediate in structure between proteins and amino acids. When serum is defecated with trichloroacetic acid, polypeptides are not precipitated, but if phosphotungstic acid is used, they are removed.[61] Therefore, by determining tyrosine in these two types of filtrates with phosphotungstic-phosphomolybdic acid, polypeptides are given by difference. This is sometimes referred to as the tyrosine index. For accuracy the concentrations of the standard and unknown must be nearly the same.[62]

An alternative is to defecate with trichloroacetic acid and then to recover the precipitate thrown down with phosphotungstic acid. This can be dissolved and polypeptides determined directly.[63]

Sample—*Serum.* To 2 ml. of sample in a tube add 8 ml. of 3 per cent trichloroacetic acid solution and mix. To another add 2 ml. of sample and 8 ml. of a solution containing 22 grams of phosphotungstic acid and 30 ml. of 1:10 hydrochloric acid per liter, and mix. After 15 minutes filter or centrifuge both and transfer 5 ml. of each solution for development.

Milk. After precipitation of casein mix 2 ml. of sample with 8 ml. of 3 per cent trichloroacetic acid. Filter and to the filtrate add 3 ml. of 10 per cent sodium phosphotungstate. Mix and add 3 ml. of 1:54 sulfuric acid. Centrifuge after 10 minutes. Wash the precipitate with phosphotungstic acid solution made by mixing 1 ml. of 10 per cent sodium phosphotungstate solution, 1 ml. of 1:54 sulfuric acid, and 3 ml. of water. Dissolve the precipitate in 2-3 drops of 1 per cent sodium hydroxide solution for development.

Procedure—*Indirect.* To the samples add 2 ml. of saturated sodium carbonate solution. Mix and add 8 drops of 5 per cent sodium cyanide solution. Add 4 drops of phosphotungstic-phosphomolybdic acid reagent (Vol. III, page 116), mix, and dilute to 10 ml. Let stand for 30 minutes and read. Subtract the value in mg. per liter for the phosphotungstic acid filtrate from that for the trichloroacetic acid filtrate to give the tyrosine index.

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[61] R. Goiffon and J. Spacy, *Bull. soc. chim. biol.* **16**, 1675-85 (1934); *Compt. rend. soc. biol.* **115**, 711-13 (1934); M. Bals and D. T. Potop, *Bull. acad. med. Roumanie* **17**, 183-6 (1945).

[62] Vintila Ciocalteu and G. Tanasesco, *Compt. rend. soc. biol.* **123**, 49-50 (1936).

[63] Etainne Goiffon, *Lait* **29**, 231-7 (1949).

Direct. Develop the solution of the phosphotungstic acid precipitate as above from ". . . add 2 ml. of saturated sodium carbonate solution . . . ."

Purine Nitrogen

Purine nitrogen after isolation is desirably converted to ammonia and estimated by Nessler's reagent.[64]

Procedure—*Urine.* Dilute 1:2 or 1:4 and use 2 ml. of sample. Add 0.5 ml. of acetic acid and 0.2 gram of sodium acetate. Heat to boiling and add 0.25 ml. of 40 per cent sodium bisulfite solution and 0.25 ml. of cupric sulfate pentahydrate solution. Heat 2 minutes longer and then centrifuge. Decant the supernatant layer to waste and add 1 ml. of wash solution containing 5 per cent of acetic acid, 0.2 per cent of copper sulfate, and 5 per cent of sodium bisulfite. Centrifuge and decant.

Digest the precipitate as for total nitrogen in blood (page 183) and develop by Nessler's reagent (page 181).

Casein

Casein in milk is determinable by the biuret reaction[65] or by development with nitric acid.[66]

Procedure—*By the biuret reaction.* Dilute the milk sample with 9 parts of water. Mix 1 ml. with 0.05 ml. of 1 per cent acetic acid solution and centrifuge. Wash the precipitate with 3 ml. and 3 ml. of ethanol, then with 3 ml. of ether. Suspend the precipitate in 1 ml. of water and add 4 ml. of biuret reagent (page 181) and let stand at 25-30° for 30 minutes. Read at 530 mμ against a reagent blank.

By nitric acid. To 1 ml. of sample, containing not more than 0.7 mg. of casein, add 6 ml. of concentrated nitric acid. Boil for 2 minutes. Cool and neutralize by slow addition of 40 per cent sodium hydroxide solution. The color changes through yellow to intense orange. Dilute to 50 ml. and mix well. Filter and read at 430 mμ within 15 minutes.

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64 T. Raekallio, *Skand. Arch. Physiol.* **81**, 1-7 (1939).

65 P. F. Fleury and R. Eberhard, *Ann. pharm. franc.* **11**, 183-7 (1953).

66 Alvarez A. Pinto and R. Carlos Medin, *Rev. quim. farm.* (Santiago Chile) **3**, No. 33, 6 (1945); cf. L. Buruiana, *Bul. soc. chim. Romania* **18A**, 191-6 (1936).

Collagen

When collagen is hydrolyzed to gelatine to make it water-soluble, it can be precipitated by tannic acid and estimated as nitrogen[67] by Nessler's reagent. The method gives high results because of other precipitable nitrogenous products.[68] A more accurate method is to estimate the separated gelatine by the hydroxyproline content.[69] When the collagen is present in only small amounts, noncollagen protein is separated from the tissue with the aid of 20 per cent urea solution. The residue after removal of collagen is appropriate for estimation of elastin.

Procedure—*Collagen content high.* Add 15 ml. of acetone to 0.1-1 gram of finely minced tissue and allow to stand for 6 hours. Decant and add 15 ml. of fresh acetone for another 6 hours. Follow with extraction by 15 ml. of ether for 12-16 hours and then dry to constant weight at 108°. This removes all fats from the tissue. If to be calculated back to the original anhydrous tissue, this must be quantitative.

To extract the collagen, accurately weigh 5-100 mg. of fat-free tissue and add to 4 ml. of water. Autoclave for 3 hours at 15 pounds' pressure. Centrifuge till clear, wash the residue with 4 ml. of water, and add to the previously decanted liquid. Autoclave the residue for 3 hours with about 4 ml. of water. Meanwhile, evaporate the supernatant liquid almost to dryness in boiling water. Centrifuge the second extraction of gelatine, wash the residue twice, and evaporate these with the first extractions. Save the insoluble residue if elastin is to be determined.

Add 1 ml. of 1:1 hydrochloric acid for about 50 mg. of extracted collagen and autoclave at 50 pounds' pressure for 3 hours. Neutralize with 10 per cent sodium hydroxide solution, dilute so that the solution contains 0.035-0.12 mg. of hydrolyzed collagen per ml., and filter. Determine hydroxyproline with *p*-dimethylaminobenzaldehyde (page 168). Read as hydroxyproline and multiply by 7.46 to convert to collagen. Correct for color contributed by other nitrogenous substances

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[67] Howard C. Spencer, Serquis Morgulis, and Violet M. Wilder, *J. Biol. Chem.* **120**, 257-66 (1937).

[68] Oliver H. Lowry, D. Rourke Gilligan, and Evelyn M. Katersky. *Ibid.* **139**, 795-804 (1941).

[69] Robert E. Neuman and Milan A. Logan, *Ibid.* **186**, 549-56 (1950).

by determination of tyrosine in the tissue extract by phosphotungstic-phosphomolybdic acid reagent (page 000).

Collagen content low. Grind 0.1-1.5 gram of fresh tissue with sand and 20 per cent urea solution. Suspend in 40-45 ml. of the 20 per cent urea solution at room temperature for 1 hour with occasional stirring. Centrifuge and discard the supernatant liquid. Wash the residue three times with 45 ml. of water, centrifuge, and discard the washings. Stir with 4-5 ml. of water and complete as for high collagen samples from "Autoclave for 3 hours at 15 pounds' pressure."

Elastin

Elastin is determined on the residue from which collogen and other soluble proteins have been separated.

Procedure—Further autoclave the residue from the collagen determination for 3 hours and wash with 8 ml. of water. Discard the extract and washings. Hydrolyze for 3 hours at 50 pounds' pressure with 1 ml. of 1:1 hydrochloric acid for each 50 mg. of residue and determine the hydroxyproline (page 168). Calculate to elastin by a figure appropriate to the source of the sample. Factors are 66.7 for pig elastin, 52.3 for beef elastin, and 43.4 for rat elastin.

CHAPTER 5

AROMATIC PRIMARY, SECONDARY AND TERTIARY AMINES [1]

This chapter follows the pattern of one of the preceding chapters in containing primary, secondary, and tertiary amines, but in this case with the amine on a benzene nucleus. Aminocyclic acids have been included in Volume III with other carbocyclic acids. Their esters are included in this chapter, partially because the esterifying agent often contains a substituted amine.

Many amines are not in this chapter or the earlier one on aliphatic amines because of a close relationship to compounds with another active group. Cycles containing nitrogen are in the following chapter, whether or not the compound also contains amines on the nucleus or side chain.

Basic reactions for amines are by diazotizing and coupling, by the coloration with *p*-dimethylaminobenzaldehyde, or by colors developed on oxidation. Differentiation between primary, secondary, and tertiary amines on diazotization depends on the primary amine forming a colorless diazo compound, the secondary amine substantially colorless nitrosamines, and the tertiary amine intensely yellow *p*-nitroso compounds.

Aniline

The diazo compound of aniline is conveniently coupled to give an intense color as the blue with 1-amino-8-naphthol-3,6-disulfonic acid, H-acid [2] or, because of greater stability, 1-amino-8-naphthol-2,4-disulfonic acid, Chicago acid.[3] The color of any tertiary amine present is compensated in a blank from which the final reagent is omitted. The reaction has been applied not only to aniline but to toluidines and naphthylamines. Therefore they interfere. As outlined, 0.05 per cent is detected and up to 6 per cent determined in the sample.

A dilute solution of aniline gives a stable yellow color with calcium

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[1] See Chapter 1, Volume III, for details of organization, condensations, etc.

[2] V. I. Minnaev, K. O. Svetlyakov and S. S. Frolov, *J. Chem. Ind. (Moscow)* **4**, 840-2 (1927); Cf. A. V. Pamfilov and M. V. Alekseeva, *Zhur. Prikladnoi Khimii* **3**, 285-9 (1930).

[3] F. L. English, *Anal. Chem.* **19**, 457-9 (1947).

hypochlorite in alkaline solution.[4] Several parts per million give an unstable purple color, which changes to yellow on dilution. Similar color is given by *o*-toluidine. The method is suitable for use below 0.06 mg. per ml. with accuracy to ±5 per cent and will detect 1 part in 2 million. In a modification, phenol is added before the hypochlorite[5] and results in a blue indophenol reaction.[5a] The phenol may also be added to an ammoniacal solution after the hypochlorite.[6]

Aniline reacts with Chloramine T in alkaline solution in the presence of sodium phenolate to give a blue color.[7] This develops to its maximum in 20 minutes and is stable for a couple of days.

Sample—*Air or other gas.* Scrub a suitable volume of gas through 100 ml. of 1:360 sulfuric acid for absorption of the aniline. If other alkaline vapors such as ammonia are present, increase the acid concentration. Add 100 ml. of 0.4 per cent sodium hydroxide solution and dilute to a known volume giving a concentration slightly below 0.05 mg. per ml. Develop by hypochlorite or Chloramine T.

Aniline in methyl or ethyl anilines. Dissolve 1 ml. of sample in 1:50 hydrochloric acid and dilute to 100 ml. with that acid. Develop the color with Chicago acid.

Yellow AB and Yellow OB. Dissolve 10 grams in 100 ml. of benzene. Extract unreacted intermediates by shaking successively at 60° with 50 ml., 25 ml., and 25 ml. of 1:19 hydrochloric acid. Wash with 50 ml. of water. Extract the combined acid extracts and washings with 25-ml. portions of benzene until such washings are no longer colored. Dilute the washed acid extract to 200 ml. for the use of aliquots for determination of aniline, *o*-toluidine, and *β*-naphthylamine by hypochlorite.

Procedure—*By Chicago acid.* Transfer 1 ml. of sample solution containing about 10 mg. of original sample to each of two 100-ml. flasks. To each add 1 ml. of 1:9 hydrochloric acid and 10 ml. of water. Cool

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[4] E. Elvove, *J. Ind. Eng. Chem.* **9**, 953-5 (1917); A. V. Pamfilov, *Ibid.* **18**, 763-4 (1926); O. L. Evenson, J. A. Kime, and S. S. Forrest, *Ind. Eng. Chem., Anal. Ed.* **9**, 74-5 (1937).

[5] Walter G. O. Christiensen, *J. Ind. Eng. Chem.* **11**, 763-4 (1919).

[5a] M. S. Bykhovskaya, *Org. Chem. Ind.* (USSR) **6**, 638-9 (1939).

[6] Fritz Micheel and Josef Schierholt, *Chem. Ber.* **85**, 1089-92 (1952).

[7] A. I. Bulycheva, *Zavodskaya Lab.* **14**, 1208-9 (1948); M. V. Alekseeva, *Ibid.* **15**, 679-80 (1949).

in an ice bath to below 5°. Add 1 ml. of a solution containing 3 per cent of sodium nitrite and 5 per cent of sodium bromide. The sodium bromide accelerates diazotization. After 1 minute in ice add 1 ml. of 10 per cent sulfamic acid solution to decompose excess nitrite. Shake in the ice bath for 30 seconds and add to one flask 2 ml. of 1 per cent solution of Chicago acid in 1:50 hydrochloric acid. To each add 10 ml. of 50 per cent sodium acetate solution and 25 ml. of methanol. The solvent dissolves nitrosamines. Dilute each to volume with water and use that to which Chicago acid was not added as the blank. Read within a few minutes.

By hypochlorite. Prepare a calcium hypochlorite solution containing about 1 per cent of available chlorine by shaking 5 grams of fresh calcium hypochlorite with 25 ml. of water for about 10 minutes and filter.

Dilute 10 ml. of acid solution of sample to 30 ml., add 3 drops of the calcium hypochlorite solution, and mix. After 2 minutes, add 1.5 ml. of 10 per cent sodium hydroxide solution, mix, and read after 10 minutes at 610 mμ against a reagent blank. If *o*-toluidine is present, it will be read as aniline.

By Chloramine T. Treat a 5-ml. neutral sample with 1 ml. of 4 per cent aqueous solution of Chloramine T. Add 1 ml. of 3 per cent sodium hydroxide solution. Dilute to 10 ml. and read against a reagent blank.

Dimethylaniline and Diethylaniline

Dimethyl- and diethylaniline are estimated as the intensely yellow *p*-nitroso compound in the presence of primary and secondary amines.[8] The vapors are absorbed in the reagent when sampling air.[9] There is no interference by aniline, methanol, or ethylene dichloride. The amines are sometimes acetylated prior to the reaction.[10] The ultraviolet absorbency of dimethylaniline in dilute ammonium hydroxide is read at 5-20 mg. per liter with accuracy to ± 1 per cent.[11]

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[8] A. S. Zhitkova, S. I. Kaplun and Joseph B. Ficklen, "Estimation of Poisonous Gases and Vapors in the Air," pp. 170-1. Service to Industry, West Hartford, Conn. (1936); F. L. English, *Anal. Chem.* **19**, 457-9 (1947).

[9] N. I. Fomicheva and P. A. Mel'nikova, *Gigiena i Sanit.* **1952**, No. 5, 49-52.

[10] J. Haslam and A. H. S. Guthrie, *Analyst* **68**, 328-30 (1943); J. Haslam and P. F. Hearn, *Ibid.* **69**, 141-5 (1944).

[11] Keith S. Heine, Jr., *J. Assoc. Official Agr. Chem.* **34**, 799-801 (1951).

Sample—*Dimethyl- or diethylanilines.* Dissolve 1 ml. in 1:50 hydrochloric acid and dilute to 100 ml. with the same acid for development as the *p*-nitroso compound.

Diethylaniline in ethylaniline. Dissolve a 5-gram sample in 10 ml. of acetic anhydride. After standing for acetylation to be complete, transfer to a distilling flask and mix with 50 ml. of water. Carefully add 10 per cent sodium hydroxide until alkaline to phenolphthalein. Distil into 1:10 hydrochloric acid and dilute the distillate to contain about 1 mg. of diethylaniline per ml. in 1:50 hydrochloric acid. Develop as the nitroso compound.

D & C Blue No. 1 and No. 2. Mix 0.5 gram of sample with 250 ml. of water, 2 grams of sodium carbonate, and a few boiling chips. Connect with two steam traps and a condenser. Distil at 2 drops per second into 1 ml. of concentrated ammonium hydroxide. When near 100 ml., dilute to that volume for reading in the ultraviolet.

Procedure—*As nitroso compound.* Mix 1 ml. of sample containing 0.1-1 mg. of aromatic tertiary amine, 1 ml. of glacial acetic acid, and 10 ml. of water. Add 5 ml. of a saturated solution of sodium nitrite and keep in the ice bath 2 minutes for dimethylaniline or 10 minutes for diethylaniline. Add 25 ml. of methanol and dilute to 100 ml. with water. Stopper, mix by inversion, and release gas pressure. Repeat 3 times and, then discard about 25 ml. of the solution. Shake the balance, releasing gas at intervals. This avoids gas bubbles on the cuvet. Read against a reagent blank.

In the ultraviolet. Read at 245 mμ against 1:100 ammonium hydroxide.

p,p',p''-Methenyltris-(N,N-dimethylaniline), Leuco Crystal Violet

The leuco form of crystal violet is oxidized to the dye with benzoyl peroxide and read.[12] Accuracy to ±0.4 per cent is obtained. Crystal violet is included in the determination, subject to correction.

Procedure—Dissolve a sample expected to contain 0.15-0.23 gram of leuco crystal violet in glacial acetic acid and dilute to 100 ml. Dilute 10 ml. of this to 100 ml. Mix 10 ml. of this with 5 ml. of 0.25 per cent solution of benzoyl peroxide in glacial acetic acid. Mix and heat in

[12] Wm. Searman, A. R. Norton, J. T. Woods, and J. J. Hugonet, *Ind. Eng. Chem., Anal. Ed.* **16**, 336-9 (1944).

boiling water for 4-5 minutes. Chill as rapidly as possible to prevent overoxidation. Dilute 5 ml. of this developed solution to 100 ml. with glacial acetic acid and read at 580 mμ within an hour. Correct for the color of an equivalent solution before oxidation.

p-Nitroaniline

The familiar diazotizing and coupling reaction is applied to *p*-nitroaniline.[13] Absence of other aromatic amines is essential.

Sample—*Air.* Flush a dry 20-liter bottle thoroughly with the gas sample. Add 60 ml. of 1:100 sulfuric acid and stopper. Shake occasionally over the next several hours to promote absorption. Transfer and flush out the bottle with water, diluting volumetrically to contain 0.5-2 mg. per ml.

Procedure—Chill a 25-ml. aliquot of the prepared sample below 5°, add 3 drops of 10 per cent sodium nitrite solution, and mix well. After 15 minutes add a fresh mixture of 6 ml. of 10 per cent sodium hydroxide solution and 3 drops of 10 per cent alcoholic β-naphthol solution. Mix well, dilute to 50 ml., and read the blue color after 20 minutes against a reagent blank.

o-Aminophenol

o-Aminophenol in *p*-aminophenol is determined by the fluorescence of the compound formed with benzoic acid.

Procedure—Dissolve a 1-gram sample in 100 ml. of 1:33 hydrochloric acid. Filter and cool. Neutralize to Congo red with concentrated ammonium hydroxide, completing dropwise with 1:15 ammonium hydroxide. Complete as for *o*-nitrophenol (Vol. III, page 124) from "Cool to room temperature and extract with 25, 25, and 25 ml. of ether" until the extract is in benzene. Wash the benzene extract with 10, 10, and 10 ml. of 1:2 hydrochloric acid and, if a residual dark color remains, wash with 5-ml. portions of 1:1 ammonium hydroxide. Wash again with 1:10 hydrochloric acid, filter through paper, and read the fluorescence.

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[13] B. V. Ponomarenko, *Zavodskaya Lab.* **8**, 996 (1939); *Ibid.* **9**, 111 (1940).

m-AMINOPHENOL

m-Aminophenol is determined by diazotizing and coupling with 2,4-dinitrophenol.[14] When present in sodium *p*-aminosalicylate it is developed with 2,6-dichloroquinonemonochloroimide.[15] Another technic for determination in sodium *p*-aminosalicylate depends on reading the diazo compound.[16] The salicylate gives a yellow diazo compound in acid solution, becomes yellow-orange when made alkaline, and fades to a faint residual yellow formed between a fraction from the diazotized salicylate and β-resorcylic acid formed in the reaction.

Procedure—*By 2,4-dinitrophenol.* Dissolve a 2-gram sample in water and dilute to 40 ml. Extract with 5 successive 30-ml. portions of ether. Wash the combined ether extracts with 10, 10, and 10 ml. of water. Discard the washings and dry the ether solution with anhydrous calcium chloride. Filter and evaporate 100 ml. of filtrate. Take up the residue in 10 ml. of water. Mix this solution with 1 ml. of 1:10 hydrochloric acid and 1 ml. of 0.5 per cent sodium nitrite solution. Chill in ice water for 5 minutes and add 1 ml. of 25 per cent urea solution. After 10 minutes add 2 ml. of 0.5 per cent 2,4-dinitrophenol in 4 per cent sodium hydroxide solution. After 10 minutes add 2.5 ml. of 1:10 hydrochloric acid and read around 500 mμ against a reagent blank.

By chloroimide. To 1 ml. of a neutral sample solution which may contain about 1 mg. of sodium *p*-aminosalicylate and containing not over 0.05 mg. of *m*-aminophenol, add 0.2 ml. of concentrated ammonium hydroxide and 0.6 ml. of 35 per cent aqueous formaldehyde. After 1 minute add 0.1 ml. of 0.1 per cent solution of 2,6-dichloroquinonemonochloroimide in butanol. Dilute to 5 ml. with water, mix, and let stand for 90 minutes. Read at 590 mμ against a reagent blank.

By diazotizing. Dilute a sample solution containing not over 1 mg. of *p*-aminosalicylate to 2 ml. Add 1 ml. of 10 per cent trichloroacetic acid and dilute to 6 ml. Chill in ice and add 0.5 ml. of 0.1 per cent sodium nitrite solution. After 5 minutes add 2 ml. of 10 per cent sodium carbonate solution and let stand for 15 minutes. Dilute to 20 ml. and read at 440 mμ against a reagent blank.

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[14] Masaru Aoki, Yoji Iwayama, and Tokinosuke Sumiyama, *J. Pharm. Soc. Japan* **71**, 1327-8 (1951); Cf. Harutado Negoro, *Ibid.* **71**, 1322-3 (1951).

[15] Atsushi Watanabe and Masaki Kamada, *Ibid.* **72**, 972-3 (1952).

[16] Ph. Jacobs, *Pharm. Weekblad* **87**, 385-96 (1952).

p-Aminophenol

When an acid solution of *p*-aminophenol is neutralized with sodium carbonate in the presence of phosphotungstic acid, a blue coloration develops as pH 7 is approached and is proportional to the amount of test substance.[17] Substantially the same color is given by hydroquinone, catechol, pyrogallol, α-naphthol, ferrous chloride, and thiosulfate. *p*-Phenylenediamines cause a red precipitate. There is no interference by phenol, cresol, aniline, β-naphthol, thymol, and salicylic acid. Both intensity and stability of color are markedly affected by the final pH. *p*-Aminophenol gives a yellow with *p*-dimethylaminobenzaldehyde in a buffered solution.[18]

Sample—*Gasoline.* Extract as described under *p*-phenylenediamine (page 227).

p-Methylaminophenol sulfate, Metol. Dissolve 0.05 gram of sample in 50 ml. of 1:10 hydrochloric acid and use 3 ml. for development with *p*-dimethylaminobenzaldehyde.

Procedure—*By phosphotungstic acid.* Mix an aliquot of acid sample solution with 2.5 ml. of phosphotungstic acid reagent (Vol. III, page 437) and add 18 per cent aqueous sodium carbonate solution with stirring until pH 7 is reached as measured with a glass electrode. Dilute to 100 ml., mix, and read at 650 mμ against a reagent blank. Centrifuge to clarify if necessary.

By p-dimethylaminobenzaldehyde. Mix a 3-ml. sample in hydrochloric acid solution with 5 ml. of ethanol. Add 10 ml. of 5 per cent *p*-dimethylaminobenzaldehyde in ethanol and 2 ml. of a solution containing 15.76 per cent of citric acid in 6 per cent sodium hydroxide solution. After 12 minutes at 20°, adjust to 25 ml. and read 15 minutes after adding the reagent. Subtract a blank.

m-Diethylaminophenol

Residual *m*-diethylaminophenol is read in the ultraviolet in analysis of certifiable dyestuffs.[19]

Sample—*D & C Red No. 19 or No. 37.* Add successively to a 500-ml. flask a boiling aid, 20 grams of monopotassium orthophosphate, and 35

[17] Lois R. Williams and Barney R. Strickland, *Anal. Chem.* **19**, 633-4 (1947).

[18] W. C. Ballard, *Analyst* **75**, 430-6 (1950).

[19] Lee S. Harrow, *J. Assocn. Official Agr. Chem.* **34**, 133-5 (1951).

grams of sodium chloride. Wash in a paste of 2.5 grams of D and C Red No. 19 or No. 37 and 50 ml. of water by successive amounts of water and dilute to 250 ml. Add 1 per cent sodium hydroxide solution until the pH is 6.6 ± 0.2. Distil through a steam trap until 175 ml. have gone over.

Procedure—Filter into 10 ml. of 10 per cent sodium hydroxide solution and dilute to 250 ml. Read at 295 mμ.

p-Ethoxyacetanilide, Acetophenetidin, Phenacetin

After acid hydrolysis, the *p*-phenetidin is oxidized by chromic acid or other strong oxidizing agents to give a ruby-red color.[20] Under properly controlled conditions this conforms to Beer's law. Acetanilide in substantial amounts gives a brown color, but this is not fully developed for several hours. An amount equal to the acetophenetidin will cause results to be 6-7 per cent high. Large amounts of acetyl *p*-aminophenol give a red color, but interference by an amount equal to the acetophenetidin is not serious. Either of these somewhat retards color development. There is no interference by acetylsalicylic acid, antipyrine, barbital, caffeine, codeine, morphine, phenobarbital, phenophthalein, phenyl salicylate, quinine, or salicylic acid. Accuracy to better than ±1 per cent is a reasonable expectation.

Phenacetin is read directly at 662 mμ.[21] Interference by acetylsalicylic acid, caffeine, codeine, or threnylpyramine can be avoided. In the ultraviolet it is read at 250 mμ without interference by aspirin but with correction necessary for caffeine.[22] An alternative technic is to warm with 10 per cent nitric acid to form 3-nitro-4-acetamidophenetole[23] which is then read. By diazotizing in glacial acetic acid, it is coupled with α-naphthol in alkaline solution to give a red color.[24]

Sample.—*Tablets.* Extract a finely ground sample containing 0.15-0.20 gram of acetophenetidin with about 50 ml. of chloroform, filter, and dilute to 100 ml. for the development of aliquots by oxidation.

[20] E. Ritsert, *Pharm. Ztg.* **33**, 383 (1888); Elmer F. Degner and Lloyd T. Johnson, *Anal. Chem.* **19**, 330-1 (1947).

[21] T. V. Parke, A. M. Ribley, E. E. Kennedy, and W. W. Hilty, *Ibid.* **23** 953-7 (1951).

[22] Marie Jones and R. L. Thatcher, *Ibid.* **23**, 957-60 (1951).

[23] Detlef Horn, *Pharm. Zentralhalle* **90**, 296-9 (1951).

[24] A. Jindra, M. Palková, and J. Zýka, *Czechoslav. farm.* **1**, 320-5 (1952).

For reading in the ultraviolet, preparation of the chloroform extract has been described under aspirin (Vol. III, page 412). A desirable content of phenacetin is 100 mg. Filter the extracts so obtained and dilute to 25 ml. with chloroform. Dilute 2 ml. to 200 ml. with chloroform.

Procedure—*By oxidation.* Combine 3 ml. of concentrated hydrochloric acid with 3 ml. of the chloroform solution of sample and warm the 2-phase system until the chloroform is evaporated and the acid volume reduced to but not below 1 ml. Take up with water and dilute to 50 ml. Mix 2 ml. of this hydrolyzed solution with 8 ml. of 50 per cent ammonium citrate solution. Add 0.1 ml. of 1 per cent aqueous chromic acid, accurately measured. Put in a bath at 23-24° for 7 minutes. Read at 543 mμ against a reagent blank.

In the ultraviolet. Read at 250 mμ and 275 mμ. Then with E calculate as follows:

$$x = 21.96E$$

	Experimental Values
$E_a = E_{1\,\text{cm.}}^{1\,\text{mg./l.}}$ for caffeine at 250 mμ	0.0131
$E_b = E_{1\,\text{cm.}}^{1\,\text{mg./l.}}$ for phenacetin at 250 mμ	0.0702
$E_c = E_{1\,\text{cm.}}^{1\,\text{mg./l.}}$ for caffeine at 275 mμ	0.0485
$E_d = E_{1\,\text{cm.}}^{1\,\text{mg./l.}}$ for phenacetin at 275 mμ	0.0159

$$xE_a + yE_b = E_m$$
$$xE_c + yE_d = E_n$$

where

E_m = absorbency of mixture at 250 mμ
E_n = absorbency of mixture at 275 mμ
x = mg. of caffeine per liter
y = mg. of phenacetin per liter

Solving for x and y and substituting standard values, the following equations are obtained:

$$x = 21.96\,E_n - 4.97\,E_m$$
$$y = 15.17\,E_m - 4.10\,E_n$$

p-Aminobenzenesulfonamide, *p*-Anilinesulfonamide, Sulfanilamide, and Related Compounds

Sulfanilamide and its successors in the sulfa drug field are subject to diazotizing and coupling with numerous reagents. They also react with such reagents as dimethylaminobenzaldehyde and β-naphthoquinone-

4-sulfonate. The general methods of determination in this topic are followed by a few designed for specific sulfa compounds.

Some readings in the ultraviolet are useful. The peak absorption by sulfanilamide is at 258 mμ and for sulfathiazole at 287.5 mμ.[25] Acetylated sulfanilamide is read at 320 mμ.[26] Sulfadiazine and sulfathiazole are determined in mixtures by reading at 239 and 280 mμ and correcting for the interference of each with the other.[27]

The familiar reaction of diazotizing and coupling with N-(1-naphthyl)ethylenediamine has been applied to sulfanilamides[28] such as *p*-aminobenzenesulfonamide and its several therapeutically active analogues. In appropriate form it is a very suitable micro method[29] for both free and total sulfanilamides. The maximum color is developed in 15 seconds and is stable for at least an hour, often 24 hours.

Sodium chloride in reasonable amounts and up to 0.5 mg. of potassium thiocyanate per ml.[30] do not interfere in the determination of 0.1 mg. of sulfathiazole per ml. Unless excess nitrite is destroyed with urea or sulfamate, high blank values may result.[31] This is corrected by the presence of ethanol in the micro method, thereby avoiding interference[32] from nitrogen bubbles. By appropriate mixtures of 0.005 per cent aqueous fuchsin and 0.00032 per cent aqueous methyl violet with water the colors can be matched as a set of permanent standards.[33]

Successful application has been made to sulfanilamide, sulfapyridine, sulfadiazine, sulfathiazole, and sulfaquinoxaline. The sensitivity of the method is improved by a factor of as much as 10 by extracting the developed solution with *n*-butanol after the azo color is produced.[34]

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25 D. T. Englis and Douglas A. Skoog, *Ind. Eng. Chem., Anal. Ed.* **15**, 748-9 (1943).

26 Scott Anderson, *Ibid.* **15**, 29-30 (1943).

27 Daniel Banes, *J. Assoc. Official Agr. Chemists* **31**, 653-5 (1948).

28 E. K. Marshall, Jr., *J. Biol. Chem.* **122**, 263-73 (1937-8); A. C. Bratton, E. K. Marshall, Jr., Dorothea Babbitt, and A. R. Hendrickson, *Ibid.* **128**, 537-50 (1939); John F. Hall, Jr., *J. Lab. Clin. Med.* **27**, 1218-22 (1942); J. S. Faber, *Pharm. Weekblad* **85**, 719-23 (1950).

29 S. W. Lee, N. B. Hannay and W. C. Hand, *Ind. Eng. Chem., Anal. Ed.* **15**, 403-6 (1943); *Science* **97**, 359-60 (1943).

30 Colin J. O. Morris, *Biochem. J.* **35**, 952-9 (1941).

31 H. Bruce Collier, *J. Lab. Clin. Med.* **30**, 285-6 (1945).

32 F. William Sunderman and D. Sergeant Pepper, and Eleanor Benditt, *Am. J. Med. Sci.* **200**, 790-5 (1940).

33 S. W. Lee and N. B. Hannay, *Ind. Eng. Chem., Anal. Ed.* **15**, 763 (1943).

34 John V. Scudi and Viola C. Jelinek, *J. Biol. Chem.* **152**, 39-40 (1944); Cf. E. Deutsch, *Schweiz. med. Wochschr.* **72**, 672-6 (1942).

Diazotized sulfanilamides may be coupled with various other amines and with phenols to give other azo dyes of varying color and tinctorial power. The colors usually range from pink to purple. Reagents so used include N-1-naphthyl-N′-diethylpropylenediamine monohydrochloride,[35] 1-(β-diethylaminoethylamino)naphthalene free from 1-naphthylamine,[36] dimethyl-α-naphthylamine,[37] ethyl-α-naphthylamine,[38] 1-acetaamido-8-naphthol-3,6-disulfonic acid,[39] 1-amino-8-naphthol-3,6-disulfonic acid,[40] N-β-sulfatoethyl-*m*-toluidine,[41] diphenylamine,[42] α-naphthylamine,[43] H acid,[44] thymol,[45] α-naphthol, and β-naphthol.[46] The diazo compound is also read directly at 380 mμ after removal of excess nitrite with urea.[47] This is equally applicable to sulfadiazine and sulfamethylthiazole.

Various reagents found satisfactory for deproteinizing for this reaction include trichloroacetic acid, *p*-toluenesulfonic acid,[48] sulfosalicylic acid,[49] and alcohol.[50] The diazo reaction requires 10 minutes

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[35] F. Nitti and Y. Joyeux, *Presse méd.* **51**, No. 6, 68 (1943); F. Servanton, *Bull. trav. soc. pharm. Bordeaux* **81**, 16-20 (1943).

[36] Gô Shimosawa and Ken'ichi Yoshida, *J. Japan Chem.* **2**, 29-31 (1948).

[37] E. K. Marshall, Jr., Kendall Emerson, Jr., W. C. Cutting and Dorothea Babbitt, *J. Am. Med. Assoc.* **108**, 953-7 (1937); E. K. Marshall, Jr., *J. Biol. Chem.* **122**, 263-73 (1937); E. K. Marshall, Jr. and J. T. Litchfield, Jr., *Science* **88**, 85-6 (1938); Asa N. Stevens and Edward J. Hughes, *J. Am. Pharm. Assn.* **27**, 36-7 (1938); H. H. van der Zoo de Jong, *Lancet* **1939, II**, 933.

[38] E. Hug, *Rev. soc. argentina biol.* **16**, 651-7 (1940); J. M. Metello Netto, *Brasil med.* **60**, 212-14 (1946).

[39] G. Oesterheld, *Schweiz. med. Wochschr.* **70**, 459-60 (1940).

[40] William B. Fortune, U. S. Patent 2,208,096 (1940).

[41] F. L. Rose and H. G. L. Bevan, *Biochem. J.* **38**, 116 (1944).

[42] John Doble and J. C. Geiger, *J. Lab. Clin. Med.* **23**, 651-3 (1938); Alfred S. Giordano and Mildred C. Prestrud, *Am. J. Clin. Path., Tech. Suppl.* **4**, 88-92 (1940).

[43] Lice Comi, *Biochim. terap. sper.* **29**, 34-9 (1942).

[44] Ts. S. Lemberg, *Gospital'noe Delo* **1946**, No. 7/8, 53-4.

[45] Gustave Deroneaux, *Arch. intern. pharmacodynamie,* **68**, 35-48 (1942); Marcel Paget, *Bull. soc. chim. biol.* **22**, 331-3 (1940); *J. pharm. chim.* **2**, 16-19 (1942); C. S. Jang and W. S. Cheng, *Chinese Med. J.* **61**, 227-32 (1943).

[46] Humiwo Tutiya and Tarō Kawamura, *Arch. Dermatol. Syphilis* **182**, 598-612 (1941); E. Havinga, *Rec. trav. chim.* **63**, 243-7 (1944).

[47] Magne Halse and Karin Wold, *Medd. Norsk. Farm. Selskap.* **13**, 103-13 (1951).

[48] E. K. Marshall, Jr., *J. Biol. Chem.* **122**, 263-73 (1937-38); E. G. Schmidt, *J. Lab. Clin. Med.* **24**, 982-5 (1939); *Ibid.* **31**, 694-9 (1946).

[49] Frank S. Schlenker, *Military Surgeon* **95**, 502-5 (1944); R. E. Florin and R. M. Silverstein, *J. Lab. Clin. Med.* **29**, 879-80 (1944).

[50] Jonas Kamlet, *Ibid.* **23**, 1101-2 (1938).

at room temperature in moderate artificial light in the presence of trichloroacetic acid,[51] and 20 minutes in the presence of toluenesulfonic acid. The coupling reaction should take place in moderate artificial light.

Procaine interferes by giving the same color.[52] When there is interference by *p*-aminosalicylic acid, it is separately determined by diazotized *p*-nitroaniline and a correction applied to the results by this method.[53] The reagents have been applied in tablet form[54] or impregnated into paper.[55] Colors developed have been compared as absorbed on white wool thread.[56] Sulfapyridine can be extracted with ether, developed, and compared against phenol red in phosphate buffers.[57]

As another type of reaction, sulfanilamides in acid solution give a yellow color with *p*-dimethylaminobenzaldehyde.[58] The tinctorial value is relatively low. Small changes in pH result in change of color intensity.[59] The reaction is given by all sulfanilamides with a free amino group such as sulfanilamide, sulfapyridine, sulfathiazole, etc. Results compare favorably with that of the diazonium salt with N(1-naphthyl)ethylenediamine dihydrochloride,[60] and with β-napthoquinone-4-sulfonate.[61] The free aldehyde of the reagent reacts with the free amino group of the sulfanilamide. Trichloroacetic acid filtrates must be neutralized because the acid inhibits full color development and excess alkali must not be present because it increases color development.[62] A buffer solution solves that. There is interference by forming the same color with free amino groups and with bilirubin, urobilin-

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[51] A. H. S. Holbourn and R. E. Pattle, *Ibid.* 28, 1028-33 (1943).

[52] Mary Frances Butler and Samuel B. Nadler, *Ibid.* 26, 1052-3 (1941); C. A. Pons and Margaret Abel, *Am. J. Clin. Path., Tech. Section* 6, 53-4 (1942).

[53] Eileen I. Short, *Biochem. J.* 48, 301-6 (1951).

[54] A. Goth, *J. Lab. Clin. Med.* 27, 827-9 (1942).

[55] John J. Engelfried, *U. S. Naval Med. Bull.* 41, 1439-44 (1943).

[56] A. Jauerneck and W. Gueffron, *Klin. Wochschr.* 16, 1544-6 (1937).

[57] Herman D. Ratish and Jesse G. M. Bullowa, *J. Lab. Clin. Med.* 25, 654-7 (1940).

[58] William V. LaRosa, *Proc. Soc. Exptl. Biol. Med.* 53, 98-100 (1943); *J. Lab. Clin. Med.* 30, 551-5 (1945); A. E. A. Werner, *Lancet* 1939, I, 18-20.

[59] Colin J. O. Morris, *Biochem. J.* 35, 952-9 (1941); Jacob Churg and David Lehr, *Am. J. Clin. Path., Tech. Suppl.* 6, 22-31 (1942).

[60] Mason C. Andrews and Arnold F. Strauss, *J. Lab. Clin. Med.* 26, 887-9 (1941).

[61] M. M. Emara and A. A. Abdelnabi, *J. Roy. Egyptian Med. Assoc.* 32, 17-23 (1949).

[62] Névio Pimenta, *O Hospital* 19, 705-13 (1941).

gen,[63] bile, and urea.[64] The color develops its full intensity at once. An alternative to photometric reading is comparison with a prestandardized 1 per cent solution of potassium chromate.

The reagent may be applied in non-oxidizing acid, in ethanol, or in 3:1 ethanol:ether.[65] The latter two are not accurate for total sulfanilamides[66] but avoid color variation with change in acid concentration and precipitation at relatively high concentrations.

The sample may be deproteinized by conventional methods. Alternatively lake a blood sample and precipitate proteins in 1:120 dilution by addition of 20 per cent of *p*-toluenesulfonic acid in 1:60 hydrochloric acid.[67] This reagent has been used by treatment of paper with a solution in hydrochloric acid and phosphoric acids, partial drying, treatment with 1 drop of serum, or blood, and comparison with standards.[68]

Reaction between amino compounds and sodium β-naphtholquinone-4-sulfonate results in deeply colored solutions or precipitates[69] which has been applied to the determination of sulfanilamide.[70] There are many constituents in protein-free blood filtrates that react with the reagent in slightly alkaline solution, but do not react in acid solution. Hence the reaction is carried out at pH 4-5. Color is developed at room temperature because higher temperatures do not accelerate it.[71] The color does not conform to Beer's law. With tungstic acid filtrates the method gives results 10-15 per cent low. Alcoholic filtrates often give higher values than aqueous solutions.

By oxidation of the sulfanilamide with a 2 per cent solution of bromine in 20 per cent sodium hydroxide solution, the intense red color is suitable for reading at least with albucid (N^1-acetylsulfanilamide),

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[63] Milton M. Hartman, *J. Lab. Clin. Med.* **26**, 401-5 (1940).

[64] D. Lehr and J. Churg, *Bull. N. Y. Med. Coll., Flower and Fifth Ave. Hosp.* **6**, No. 2, 59-69 (1943).

[65] Abraham Saifer, *Bull. U. S. Army Med. Dept.* **4**, 610-12 (1945).

[66] S. W. Lee and N. B. Hannay, *J. Am. Pharm. Assoc.* **32**, 307-8 (1943).

[67] Colin J. O. Morris, *Biochem. J.* **35**, 952-9 (1941); Raymond W. Monto, *Am. J. Clin. Path., Tech. Suppl.* **5**, 165-7 (1941).

[68] Wm. V. LaRosa, *Proc. Soc. Exptl. Biol. Med.* **53**, 98-100 (1943); *J. Lab. Clin. Med.* **30**, 551-5 (1945).

[69] O. N. Witt, *Ber.* **24**, 3154 (1891); M. Böniger, *Ibid.* **27**, 23 (1894); C. A. Herter, *J. Exp. Med.* **7**, 79 (1905); Otto Folin, *J. Biol. Chem.* **51**, 377 (1922).

[70] E. G. Schmidt, *Ibid.* **122**, 757-62 (1937-38); *J. Lab. Clin. Med.* **23**, 648-50 (1938); *Ibid.* **24**, 795-8 (1939).

[71] E. G. Schmidt, *Am. J. Clin. Path., Tech Sect.* **10**, 153-5 (1946).

sulfathiazole, sulfamethylthiazole, sulfapyridine, sulfaguanidine, 4'-sulfamyldsulfanilamide, uliron (N^1,N^1-dimethyl-N^4-sulfanilylsulfanilamide) and the sodium salt of sulfasuccinalic acid.[72] Negative results are obtained with neostreptosil and *p*-sulfamylacetanilide. Reaction with furfural and acetic acid can be used but is relatively insensitive.[73] Sulfonamides are also developed with sodium nitroprusside and potassium permanganate in alkaline solution.[74]

Samples—*Blood*.[75] Prepare sulfuric-trichloroacetic acid mixture by adding 56 ml. of 1:8 sulfuric acid to 1 liter of 3.33 per cent trichloroacetic acid. This is preferred to trichloroacetic acid for deproteinizing. Add 0.3 ml. of whole blood dropwise to 5.7 ml. of sulfuric-trichloroacetic acid mixture. Break up each drop with vigorous stirring and agitate after all the blood has been added. Allow to stand until precipitated protein settles and filter to collect at least 4 ml. of filtrate for development with N-(1-naphthyl)ethylenediamine as the sulfuric-trichloroacetic acid filtrate.

For development with dimethyl-*α*-naphthylamine, mix 2 ml. of blood containing 0.5 gram of sodium fluoride with 16 ml. of 0.05 per cent aqueous saponin solution and let stand for 2 minutes to allow complete hemolysis. Add 2 ml. of 50 per cent trichloroacetic acid solution dropwise, let stand for 5 minutes, and filter.

For development with ethyl-*α*-naphthylamine, mix 1 ml. of sample with 9 volumes of absolute ethanol to deproteinize. Filter and make the filtrate acid with 1:10 hydrochloric acid.

For development with *α*-naphthol,[76] mix 2 ml. of sample with 14 ml. of water and 4 ml. of 20 per cent trichloroacetic acid solution. Filter and develop.

For development with thymol[77] add 0.5 ml. of water to 0.002 ml. of blood. Precipitate proteins with a drop of saturated alum solution to which a pinch of calcium carbonate has been added. Heat for 10 minutes in boiling-water and refill with water to the former mark. Filter and wash the precipitate with 0.5 ml. of water.

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[72] Enrico Cignolani, *Boll. chim. farm.* **86**, 143-6 (1947).

[73] V. Arreguine, *Anales asoc. quím. argentina* **30**, 39 (1940).

[74] Antonio Esposito Vitolo, *Boll. chim. farm.* **89**, 351-60 (1950).

[75] S. W. Lee, N. B. Hannay, and W. R. Hand, *loc. cit.*

[76] P. Fantl, *Australian J. Exptl. Biol. Med. Sci.* **18**, 175-84 (1940).

[77] A. V. Stepanov and V. V. Mamaeva, *Klin. Med. (USSR)* **24**, 76-7 (1946).

For development of total sulfonamides with *p*-dimethylaminobenzaldehyde,[78] add 5 ml. of oxalated blood dropwise to 20 ml. of 5 per cent trichloroacetic acid solution, shake, and filter. For free sulfonamides add 0.5 ml. of blood to 6.5 ml. of water, mix, and leave for 3 minutes. Add 0.5 ml. of 30 per cent salicylsulfonic acid solution and shake vigorously. After 10 minutes filter through double paper.

Urine. For development with N-(1-naphthyl)ethylenediamine, dilute 0.1, 0.2, or 0.3 ml. to 6 ml. with sulfuric-trichloroacetic acid mixture.

For development with dimethyl-α-naphthylamine, dilute 1 ml. of urine to about 8 ml., add 1 ml. of 50 per cent trichloroacetic acid solution, dilute to 10 ml., and filter.

For development with ethyl-α-naphthylamine,[79] mix equal volumes of urine and 40 per cent sodium hydroxide solution, and add 8 volumes of 1:10 hydrochloric acid solution. Filter before use.

Bile.[80] Add 3 ml. of 11.2 per cent potassium hydroxide solution to 5 ml. of bile containing more than 0.02 mg. of sulfonamide per ml. Add 3 ml. of 40 per cent zinc sulfate solution dropwise with constant agitation. To insure solution of the drug, add portions of hot water. Dilute to approximately 45 ml., add 3 ml. of 14 per cent potassium carbonate solution, and a drop of phenolphthalein solution. Cool, dilute to 50 ml., and filter. Acidify the filtrate to phenolphthalein with 2-3 drops of 1:2 hydrochloric acid. Use 10 ml. of filtrate for development of free or total sulfanilamides with N-(1-naphthyl)ethylenediamine.

Cerebrospinal fluid. Use 10 ml. as sample for development with N-(1-naphthyl)ethylenediamine.

Feces.[81] Extract the sample with 8 per cent sodium hydroxide and after 24 hours acidify the extracts with 1:1 hydrochloric acid. Dilute an aliquot to 10 ml. with water, add 3 ml. of 1:5 hydrochloric acid, and filter. Develop with ethyl-α-naphthylamine.

Procedure—*By N-(1-naphthyl)ethylenediamine. Free sulfanilamides.* Add 0.1 ml. of 0.1 per cent sodium nitrite solution to exactly 2 ml. of sulfuric-trichloroacetic acid filtrate and allow to diazotize for 3 minutes. Add 1 ml. of ethanol and swirl. Add 0.1 ml. of 0.1 per

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[78] Wm. W. Williams, *Rocky Mt. Med. J.* **37**, 110-11 (1940).

[79] G. Hecht, *Dermat. Wochschr.* **106**, 261-3 (1938).

[80] H. M. Carryer and A. E. Osterberg, *J. Lab. Clin. Med.* **28**, 110-12 (1942).

[81] C. C. Jensen and K. Pedersen-Bjergaard, *Dansk Tids. Farm.* **17**, 199-208 (1943).

cent aqueous N-(1-naphthyl)ethylenediamine dihydrochloride solution and read at 545 mμ against a reagent blank after 1 minute.

Total sulfanilamides. Heat 2 ml. of acid filtrate for 1 hour in boiling water. Cool, adjust the volume to 2 ml. with water, and continue as for free sulfanilamides.

Butanol extraction of developed colors. To concentrate the color, 5 minutes after coupling extract the developed sample with 3 ml. of *n*-butanol. Separate the butanol layer, chill in ice water, and centrifuge out any moisture cloud. Read at 540 mμ. Extraction is only 90 per cent complete with sulfanilamide itself but complete with heterocyclic sulfanilamides.

By dimethyl-a-naphthylamine.[82] *Free sulfonamide. Urine.* To 1 ml. of sample add successively 9 ml. of water, 1 ml. of 50 per cent trichloroacetic acid solution, and 1 ml. of fresh 0.1 per cent sodium nitrite solution. Shake and let stand for 2 minutes. To destroy excess nitrite, dissolve 1 gram of urea in 5 ml. of water and dilute to 100 ml. with acetone as 1 per cent urea in acetone. Alternatively use 0.5 per cent of sulfamic acid neutralized with ammonia in 13.8 per cent monosodium phosphate solution. Add 2 ml. of one of these reagents, shake again, and allow to stand for another 2 minutes. Add 1 ml. of 0.4 per cent dimethyl-α-naphthylamine in a 1:1 alcohol-acetone. Shake and add 8 grams of ammonium sulfate crystals. Shake and allow the layers to separate and read the upper layer at 500-540 mμ [83] against a reagent blank.

Blood. Mix 2 ml. of blood containing 0.5 gram of sodium fluoride and add 16 ml. of 0.05 per cent saponin solution. After 2 minutes for hemolysis add, drop by drop, 2 ml. of 50 per cent trichloroacetic acid solution. Filter after 5 minutes. To 10 ml. of filtrate add 1 ml. of water and 1 ml. of fresh 0.1 per cent sodium nitrite solution. After 2 minutes add 3 ml. of the 1 per cent solution of urea in acetone, described under urine. Shake and complete as for free sulfonamides in urine from "After 2 minutes add 1 ml. of . . .."

Total sulfonamide. Urine. To a 5-ml. sample add 2 ml. of 1:9 sulfuric acid and 3 ml. of water. Heat in boiling water for 30 minutes, cool, and neutralize to litmus with 10 per cent sodium hydroxide solution.

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[82] L. Servantie and G. Demange, *Bull. trav. soc. pharm. Bordeaux* 78, 102-11 (1940); R. Fabre and J. Hagopian, *Ann. pharm. franç.* 2, 126-8 (1944).

[83] L. Servantie and G. Demange, *J. pharm. chim.* 30, 62-70 (1939).

Dilute to an appropriate volume and use a 1-ml. aliquot as described for free sulfonamide.

Blood. Carry on for free sulfonamide through "Filter after 5 minutes." Neutralize 5 ml. of the filtrate with 10 per cent sodium hydroxide solution and acidify with 1:9 sulfuric acid. Dilute to 10 ml. and heat in boiling water for 30 minutes. Neutralize with 10 per cent sodium hydroxide solution and dilute to 10 ml. again. Add 1 ml. of water and 1 ml. of fresh 0.1 per cent sodium nitrite solution. Complete as for free sulfonamides in urine from "After 2 minutes add 1 ml. of . . .."

By ethyl-α-naphthylamine. To a 1-ml. aliquot add 0.1 ml. of 1 per cent sodium nitrite solution. After 3 minutes add 1 ml. of 0.2 per cent aminosulfonic acid solution or 0.2 per cent urea solution, followed by 1 ml. of 0.3 per cent ethyl-α-naphthylamine hydrochloride solution. Dilute to 10 ml. with methanol and read at 530 mμ against a reagent blank. Turbidity may result in an ethanol filtrate upon addition of the acid due to partial precipitation of lipoids.[84] To avoid this add 2 ml. of diethylether to the sample after the reagent and shake vigorously.

By α̃-naphthol. To 4 ml. of sample, add 0.4 ml. of 0.5 per cent sodium nitrite solution. Allow to stand at 10° for 5 minutes and add 2 ml. of 4 per cent sodium hydroxide solution and 0.5 ml. of 0.5 per cent α-naphthol solution. Let stand and read after 30 minutes at 480-546 mμ against a reagent blank.

By thymol. To 1 ml. of sample add 2 drops of 1:2 hydrochloric acid and 1 drop of 0.1 per cent sodium nitrite solution. After 10 minutes at 30°, add 4 drops of a 10 per cent sodium hydroxide solution and 4 drops of 2 per cent thymol in 5 per cent sodium hydroxide solution. After 10 minutes at 30° filter through cotton and read against a reagent blank at 500 mμ.[85]

By p-*dimethylaminobenzaldehyde.* As reagent, disperse 7 grams of *p*-dimethylaminobenzaldehyde in 7:93 sulfuric acid by shaking and dilute to 100 ml. with this acid. Decolorize with 0.3 gram of activated carbon and then filter.

Total sulfanilamides.[86] Add 0.5 ml. of sulfuric acid to 2 ml. of the sample and heat gently on a water bath for 40 minutes. Cool and add

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84 H. G. Piper, *Klin. Wochschr.* 20, 152 (1941).

85 José Antonio Salvá Miguel, *Trabajos inst. nacl. cienc. méd.* (Madrid) 3, 439-48 (1943-4).

86 Colin A. Mawson, *Biochem. J.* 36, 845-7 (1942).

2.5 ml. of water. Develop the color by addition of 0.5 ml. of the reagent to 2 ml. of filtrate and read at 420 mμ against a reagent blank.

Free sulfonamides. To 2 ml. of a specially deproteinized sample add 0.5 ml. of reagent and read.

By sodium β-naphthoquinone-4-sulfonate. To 10 ml. of sample containing 0.02-0.2 mg. of sulfanilamide, add 1 drop of 1:120 hydrochloric acid. Add 1 ml. of freshly prepared 0.05 per cent solution of sodium β-naphthoquinone-4-sulfonate. Mix well and place in the dark at once for 45-60 minutes. Read against a reagent blank.

N¹-Acetylsulfanilamide, Albucid

Determine by general methods described on page 205 *et seq.*

N¹,N¹-Dimethyl-N⁴-sulfanilylsulfanilamide, Uliron

Determine by general methods described on page 205 *et seq.*

p-(Aminomethyl)benzenesulfonamide, Homosulfanilamide

This sulfanilamide is developed with 1,2-naphthoquinone-4-sulfonic acid.[87]

Procedure—Mix 5 ml. of sample containing 0.001-0.01 mg. of homosulfanilamide with 5 ml. of 0.05 per cent 1,2-naphthoquinone-4-sulfonic acid solution. Add 30 per cent sodium hydroxide solution until distinctly alkaline and keep at 4° for 30 minutes. Read the yellow color against a reagent blank.

Sulfathiazole

In mixtures of sulfathiazole, sulfadiazine, and sulfamerazine in the same tablet determine sulfathiazole by the yellow color on diazotizing.[88] There is no interference by the other two sulfa drugs present or by acetosulfamine or sulfanilamide. Sulfamethylthiazole produces a deep yellow color which fades after 20 minutes. Sulfapyridine interferes slightly.

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87 Tsutomu Momose and Tomohiro Yasumura, *J. Pharm. Soc. Japan* **70**, 672 (1950).

88 Massaru Aoki and Yoji Iwayama, *Japan. J. Pharm. and Chem.* **22**, 252-7 (1950).

In 1:15 hydrochloric acid the maximum absorption is about 280 mμ. Sulfadiazine and sulfamerazine do not interfere. For other pertinent reactions see page 205 *et seq.*

Procedure—Adjust the acidity of 2 ml. of sample to approximately 1:10 with hydrochloric acid. Add 0.1 ml. of 5 per cent sodium nitrite solution, adjust to a known volume and read.

Sulfamethylthiazole

The reaction with *p*-dimethylaminobenzaldehyde is applicable to sulfamethylthiazole.[89] There is no interference by sulfathiazole, sulfadiazine, sulfamerazine, or acetosulfamide. The diazo compound is also read at 380 mμ after removal of excess nitrite with urea.[90] Sulfanilamide and sulfadiazine interfere. Other pertinent reactions are discussed under sulfanilamide (page 205 *et seq.*).

Procedure—Add concentrated hydrochloric acid and water to the sample until it is adjusted to 0.5-5 mg. of sulfamethylthiazole per ml. in 1:10 hydrochloric acid. To 5 ml. of such sample add 0.1 ml. of 10 per cent *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid. Mix and heat in boiling water for 90 minutes. Cool and read.

N^1-(5-Ethyl-1,3,4-thiodiazol-2-yl)sulfanilamide, Sulfa-2-ethylthiadizole

This is read if sulfa-2-ethylthiodiazole is present; read it in 1:15 hydrochloric acid at 268 mμ.[91] Sulfadiazine and sulfamerazine do not interfere, but sulfathiazole does.

Sulfadiazine

Sulfadiazine reacts with thiobarbituric acid to give a color arising from the pyrimidine structure.[92] It follows that the other sulfa drugs

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89 Masuru Aoki and Yoji Iwayama, *Japan J. Pharm. and Chem.* **22**, 252-7 (1950).

90 Magne Halse and Karin Wold, *Medd. Norsk. Farm. Selskap.* **13**, 103-13 (1951).

91 Jacques Thomas and Gaston Lagrange, *J. Pharm. Belg.* **6**, 355-66 (1951).

92 Masaru Aoki and Yoji Iwayama, *Japan. J. Pharm. and Chem.* **22**, 252-7 (1950).

do not interfere. An alternative is to read the diazo compound a 380 mμ after removal of excess nitrite with urea.[93] Sulfathiazole an sulfamethylthiazole interfere.

A red color can also be developed by vanillin in sulfuric acid solutio on heating. The intensity of color is affected by the sulfuric acid con centration. An initial yellowish green from the other sulfa drugs fade on heating.

Sulfadiazine and sulfamerazine can be developed by resorcinol and read in the same solution.[94] For general reactions of sulfanilamides see page 205 *et seq.*

Procedure—*By vanillin.* Adjust the acidity of a 2-ml. sample con taining 0.001-1 mg. of sulfadiazine per ml. to 1:10 with hydrochloric acid. Add to 3 ml. of 1 per cent aqueous sodium thiobarbiturate. Hea in boiling water for 1 hour, cool, and read against a reagent blank.

By resorcinol. To a 2-ml. sample add 2 ml. of concentrated sulfuric acid, then dropwise 1 ml. of fresh aqueous 5 per cent resorcinol. Mix chill in cold water, and place in an 80° bath for 30 minutes. Chill in ice and dilute to 10 ml. with a fresh cold 1:1 mixture of 3:2 sulfuric acid and glycerol. Ten minutes after removal from the bath read at 440 mμ for sulfadiazine. Fifteen minutes after removal from the bath read at 320 mμ for sulfamerazine. As blank use 2 ml. of concentrated sulfuric acid, 1 ml. of water, and the sulfuric acid-glycerol mixture to dilute to 10 ml.

By thiobarbituric acid. Use the general method for pyrimidines (page 293).

Sulfamerazine

Sulfamerazine is determined by its red color with orcinol aldehyde.[95] There is no interference by sulfathiazole, sulfanilamide, sulfadiazine, or acetosulfamine. It is developed with resorcinol and read in the presence of sulfadiazole by a method given under the latter (above). For other reactions see page 205 *et seq.*

[93] Magne Halse and Karin Wold, *Medd. Norsk Farm. Selskap.* **13**, 103-13 (1951).

[94] Jasques Thomas and Gaston Lagrange, *J. pharm. Belg.* **6**, 355-66 (1951).

[95] Masaru Aoki and Yoji Iwayama, *Japan. J. Pharm. and Chem.* **22**, 232-7 (1950).

N[1]-Guanylsulfanilamide, Sulfaguanidine

Sulfaguanidine is a sulfa drug used in poultry feeds. It is most conveniently determined by N-(1-naphthyl)ethylenediamine.[96] For further discussion see page 205 *et seq.*

Sample—*Poultry feed.* Stir a 1-gram sample with 100 ml. of 1:200 hydrochloric acid and bring cautiously to a boil. Dilute to 250 ml. and let settle. Mix 50 ml. with 18 ml. of 15 per cent trichloroacetic acid and dilute to 100 ml. Filter, rejecting the first 10 ml., and dilute 10 ml. to 100 ml. as sample.

Procedure—Stir 5 ml. of clear solution expected to contain 0.01-0.05 mg. of sulfaguanidine with 5 ml. of 6 per cent trichloroacetic acid and 1 ml. of fresh 0.1 per cent sodium nitrite solution. After 3 minutes add 1 ml. of 0.5 per cent ammonium sulfamate, and let stand for 2 minutes. Add 1 ml. of a 0.1 per cent solution of N-(1-naphthyl)ethylenediamine dihydrochloride and let stand for 10 minutes. Read at 525 mμ against a reagent blank and subtract the absorption of a 5-ml. sample diluted to 13 ml.

N[1]-(2-Quinoxalyl)sulfanilamide, 2-Sulfanilamidoquinoxaline, Sulfaquinoxaline

Sulfaquinoxaline is a sulfanilamide in which sulfanilic acid is condensed with 2-aminoquinoxaline. It shows the same color reactions as sulfanilamide and related compounds. Use is particularly in poultry feeds.[97] The development of color with N-(1-naphthyl)ethylenediamine dihydrochloric is satisfactory, giving the characteristic band at 545 mμ which sulfanilamide does. The color developed does not conform to Beer's law. For further discussion see page 205 *et seq.*

Samples—*Salts.* Dissolve a 0.1-gram sample in 5 ml. of 2 per cent sodium hydroxide solution and dilute to 100 ml. Dilute an appropriate aliquot of this to 50 ml. and use 2 ml. of that dilution as sample. Thus for a preparation approaching purity, the aliquot for further dilution is 4 ml. Insolubles may be allowed to settle out before aliquoting.

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96 Stanley W. Tyler, Richard O. Brooke, and Warren S. Baker, *Ind. Eng. Chem., Anal. Ed.* 21, 1091-2 (1949).

97 S. W. Tyler, R. O. Brooke, and W. S. Baker, *Anal. Chem.* 21, 1091-2 (1949); James P. Dux and Charles Rosenblum, *Ibid.* 21, 1524-7 (1949).

Poultry feed. The data on sample weight, etc., are as follows:

Percentage Sulfaquinoxaline	*Weight of Sample*	*Aliquot for Dilution*	*Aliquot for Development*
0.01- 0.1	5	50	10-15
0.1 - 0.5	2	25	10
0.5 - 1.0	1	25	10
1.0 - 5.0	1	10	4
5.0 -15.0	1	10	2

Mix the weighed sample with 100 ml. of water and add 2 per cent sodium hydroxide solution in 1-ml. increments until alkalinity to litmus paper is present. Boil gently for about 2 minutes and cool to room temperature. Dilute to 250 ml. and remove an aliquot as indicated in the table. Add 10 ml. of 1 per cent zinc sulfate solution and dilute to 100 ml. Mix and filter the deproteinized solution, discarding the first 10 ml.

Alternatively if necessary to expedite filtration, heat the solution and precipitate to 60-70° for 2 minutes and let stand for 10 minutes. Filter the supernatant liquid from which the coagulum will have separated and discard the first 10 ml. of filtrate. Use an aliquot according to the table for development.

Procedure—Measure an aliquot of sample containing 0.05-0.35 mg. of sulfaquinoxaline and, if strongly alkaline, approximately neutralize with 1:25 hydrochloric acid. Add 2.5 ml. of accurately prepared 0.5 *N* hydrochloric acid and 2 ml. of 0.1 per cent sodium nitrite solution prepared fresh daily. After 3 minutes add 2 ml. of 0.5 per cent ammonium sulfamate solution. After 2 minutes add 2 ml. of 0.1 per cent N-(1-naphthyl)ethylenediamine dihydrochloride solution from storage in a dark bottle. Dilute to 50 ml. with water. The final pH should be 1.3-1.4. Read at 545 mμ against a reagent blank from 0.5-15 minutes after coupling. If turbidity interferes, subtract the reading of a sample from which nitrite and sulfamate have been omitted.

Sulfapyridine

Determine by general methods described on page 205 *et seq.*

Ethyl Ester of *p*-Aminobenzoic Acid, Benzocaine

Determine as described for procaine with N-(1-naphthyl)ethylenediamine dihydrochloride.

Butyl Ester of p-aminobenzoic acid, Butesin

Determine as described for procaine with N-(1-naphthyl)ethylenediamine dihydrochloride.

Diethylaminoethyl Ester of p-Aminobenzoic Acid, Procaine, Novocaine, Ethocaine

The usual methods of determination of procaine depend on the aromatic amine group. It is therefore not surprising that many methods are similar to those for p-aminobenzoic acid. Procaine may also be hydrolyzed to that acid for determination.

When diazotized and coupled with β-naphthol, the red color can be read to ± 2 per cent.[98] Similarly it is coupled with N-(1-naphthyl) ethylenediamine hydrochloride.[99] Sulfanilamide does not interfere.

Other reactions which have been used are addition of ammonia to the solution of diazonium chloride to give a yellow,[100] the yellow condensation product with vanillin,[101] the red to yellow with potassium guaiacol sulfonate,[102] and the red with 1-amino-8-naphthol-3,6-disulfonic acid to give a red color, sensitive to 0.027 mg. per ml.[103] An acid medium is used, but the speed of oxidation must be controlled. When reacted with p-methylaminobenzaldehyde in absolute ethanol and concentrated sulfuric acid the reaction determines 0.008 mg. per ml.[104] After diazotizing and coupling with 1-sulfomethylaminonaphthalene-8-sulfonic acid, it is read at 550 mμ.[105] It is separated and read as the reineckate by a method described under xylocaine (page 224). Procaine in procaine penicillin is read directly at 290 mμ,[106] which is one of the

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[98] Walter Eissner, *Arch. Pharm.* **268**, 322-3 (1930); Herbert Seydlitz, *Svensk. Farm. Tid.* **50**, 65-70 (1946); Charles Lapiere, *Anal. chim. acta* **1**, 337-40 (1947).

[99] Bruno Kisch and Eduard Strauss, *Expt. Med. and Surg.* **1**, 61-70 (1943); F. J. Bandelin and C. R. Kemp, *Ind. Eng. Chem., Anal. Ed.* **18**, 470-1 (1946); Kuang S. Ting, Julius M. Coon, and Alvin C. Conway, *J. Lab. Clin. Med.* **34**, 822-9 (1949).

[100] E. Raymond Riegel and John F. Williams, *J. Am. Chem. Soc.* **48**, 2871-8 (1926).

[101] Harry Koster, Arthur Shapiro, and Edna Posen, *J. Lab. Clin. Med.* **21**, 1096-8 (1936); G. F. Reikhardt, *J. Applied Chem.* (USSR) **11**, 387-8 (1938).

[102] P. Cheramy, *J. pharm. chim.* **30**, 408-11 (1924).

[103] Harry Willstaedt, *Biochem. Z.* **269**, 182-6 (1934); E. E. Vonesch and O. A. Guagnini, *Arch. farm. bioquím. Tucumán* **4**, 317-19 (1950).

[104] Masaji Yamagishi, *Ann. Rept. Takeda Research Lab.* **10**, 35-9 (1951).

[105] P. Terp, *Acta Pharmacol. Toxicol.* **6**, 269-84 (1950).

[106] C. V. St. John, *J. Am. Pharm. Assn., Sci. Ed.* **37**, 343-4 (1948).

maxima for procaine hydrochloride.[107] Another is at 221 mμ. Minima are at 240 mμ and 209 mμ.

Samples—*General.* Dilute to an approximate concentration of 50 mg. per liter. If sodium bisulfite is present as a preservative, add 1 drop of 1.27 per cent iodine solution for each 5 ml. of sample. Develop with any of the reagents.

Tissue. Mince 2-5 grams of tissue and rub up with 2 grams of sodium bicarbonate to neutralize acidity present. Add 5 grams of anhydrous sodium sulfate and grind. Add two further 5-gram portions and grind with each addition. This gives a crumbly mass. Transfer to a Soxhlet thimble and clean out the mortar with more anhydrous sodium sulfate. Extract with ether for one hour and evaporate the ether from the extract. Treat the residue with 1 ml. of 1:3 hydrochloric acid and filter. Repeat the treatment of the residue twice more. Use the combined filtrates as sample for diazotizing and coupling.

Blood. Separation in the presence of *p*-aminobenzoic acid was described under that topic (Vol. III, page 417). The final extraction is in aqueous acid solution for development with N-(1-naphthyl)ethylenediamine dihydrochloride.

Urine. To an appropriate sample add 0.5 ml. of 10 per cent sodium carbonate solution. Extract mechanically for 2 minutes with 10 ml. of ether. Separate and extract with another 10 ml. of ether. Add 5 ml. of 1:15 hydrochloric acid to the combined ether extracts, shake and separate the aqueous acid layer. Dilute to a known volume and develop an aliquot with N-(1-naphthyl)ethylenediamine dihydrochloride.

Barbital solutions. Mix 5 ml. of solution with 2 ml. of 1:6.5 ammonium hydroxide. Add 20 ml. of a 3:1 mixture of chloroform and isopropanol, shake, and separate. Extract twice more. Evaporate the extract and take up in 25 ml. of acetone for development of aliquots with β-naphthol. *p*-Aminobenzoic acid is in the aqueous alkaline layer. Its determination appears under that topic (Vol. III, page 418).

Pomades. Dissolve a weighed sample in 20 ml. of petroleum ether. Shake the filtered solution with 5 ml. of 1:25 hydrochloric acid. Separate the acid layer and develop all or an aliquot with β-naphthol.

Injection solutions. Dilute to approximately 0.01 mg. of test substance per ml. for the use of aliquots.

[107] A. I. Biggs, *J. Pharm. and Pharmacol.* **4**, 479-84 (1952).

Ointments. Dissolve a weighed sample expected to contain 0.1 gram of test substance in 50 ml. of petroleum ether. Extract with five 10-ml. portions of 1:100 sulfuric acid. Filter the combined extracts through a wet paper and dilute to 100 ml. with water. Dilute 5 ml. to 500 ml. for the use of aliquots.

Tablets. Suspend or dissolve a weighed portion of the ground tablets expected to contain 0.1 gram of test substance in 50 ml. of distilled water. Make alkaline with 1:3 ammonium hydroxide and extract with five 10-ml. portions of chloroform. Filter the combined extracts through a pad of cotton wet with chloroform and wash the pad with 10 ml. of chloroform. Evaporate to about 5 ml. on the water bath in a stream of air. Add 5 ml. of ethanol and continue to evaporate until dry. Take up the residue in 5 ml. of ethanol, add 50 ml. of 1:100 sulfuric acid, and warm at 50° to dissolve. Dilute volumetrically with water to 100 ml. and dilute 5 ml. of this to 500 ml. for the use of aliquots.

Procaine penicillin.[108] Dissolve 50 mg. of procaine penicillin G in nearly 100 ml. of water by shaking and dilute to 100 ml. Dilute a 5-ml. aliquot to 250 ml. and read in the ultraviolet.

Procedure—*By β-naphthol.* Mix 1 ml. of sample solution containing 0.1-0.6 mg. of procaine, 0.2 ml. of 1 per cent sodium sulfite solution, and 1 ml. of 1:10 hydrochloric acid solution. Shake and after a few minutes add 1 ml. of 1 per cent acetone solution of β-naphthol and 2 ml. of 8 per cent sodium hydroxide solution. Shake until the red color develops. This reaches a maximum within 3 minutes. The sodium hydroxide salts out the acetone with the color dissolved in it. Let stand to separate and remove the acetone layer. Dilute to 25 ml. and read at 500 mμ within 1 hour after adding the naphthol.

With N-(1-naphthyl)ethylenediamine dihydrochloride. To a 5-ml. aliquot of prepared solution containing 0.01-0.05 mg. of test substance add 5 ml. of 1:8 sulfuric acid. Mix in 1 ml. of 0.1 per cent sodium nitrite solution and, after 3 minutes, 5 ml. of ethanol. After 2 minutes add 1 ml. of 0.1 per cent solution of N-(1-naphthyl)ethylenediamine and dilute with water to 50 ml. Read at 545 mμ against a reagent blank.

In the ultraviolet. Read at 290 mμ against the menstruum of the sample.

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[108] C. V. St. John, *J. Am. Pharm. Assn., Sci. Ed.*, **37**, 343-4 (1948).

Dibutylaminoethyl Ester of *p*-Aminobenzoic Acid, Butyn, Butacaine

Determine as described for procaine with N-(1-naphthyl)ethylenediamine dihydrochloride.

2-Dimethylaminoethyl ester of *p*-butylaminobenzoic acid, Pantocaine, Tetracaine

Tetracaine hydrochloride is determined, after separation from its hydrolytic product, *p*-butylaminobenzoic acid, by reading at 310 mμ and pH 6-8.[109] Phenylephrine hydrochloride does not interfere. The method is accurate to ±3 per cent. An alternative is nitration to give the characteristic aromatic nitro color in ketones.[110]

Sample—*Solutions, tablets, concentrates.* Prepare an aqueous solution to contain approximately 0.5 mg. of tetracaine hydrochloride with or without 0.5 mg. of phenylephrine hydrochloride per ml. Dilute 50-fold with 1:1500 hydrochloric acid. Extract a 20-ml. aliquot twice with 25-ml. portions of water-saturated benzene, to separate *p*-butylaminobenzoic acid. Filter the aqueous layer and neutralize an aliquot of the filtrate with an equal volume of 0.04 per cent sodium hydroxide solution.

Procedure—*In the ultraviolet.* Read at 310 mμ, at which wave length phenylephrine gives no measurable absorption.

By nitration. Evaporate a sample containing 0.1-0.4 mg. of tetracaine to dryness. Cool and moisten the residue with 0.2 ml. of fuming nitric acid. Heat on a water bath for 5 minutes and take up in 10 ml. of acetone. Add 5 ml. of water and dilute to 25 ml. with acetone. Read at 550 mμ against a reagent blank.

Diethylaminoethyl Diphenyl Acetate

The solution of the nitro compound of diethylaminoethyl diphenylacetate is suitable for its estimation.[111] The color is developed in alkaline solution and is only stable over the period 2 to 6 minutes.

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[109] Robert I. Ellin and Albert A. Kondritzer, *J. Am. Pharm. Assoc.* **41**, 71-4 (1952).

[110] Herbert Seydlitz, *Svensk Farm. Tid.* **50**, 593-6 (1946); Teodor Canbäck, *Svensk Kem. Tid.* **58**, 101-3 (1946).

[111] Teodor Canbäck, *Farm. Rev.* **45**, 377-80, 617-18 (1946).

Sample—Acidify 0.05 gram suspended in 100 ml. of water by addition of a drop of 1:1 hydrochloric acid. Filter and dilute the filtrate to 200 ml.

Procedure—To 4 ml. of sample add 1 ml. of 10 per cent sodium carbonate solution. Extract successively with 10, 5, and 5 ml. of ether. Dilute the extract to 25 ml. and evaporate 2 ml. to dryness. Nitrate on a steam bath with 10 drops of fuming nitric acid and remove the excess *in vacuo*. Take up the residue in 98:2 absolute acetone and ethanol. Dilute to 10 ml. with that solvent and add 5 drops of 3 per cent potassium hydroxide in methanol. Read after 2 minutes at 570 mμ against a reagent blank.

N-Dimethylaminoethyl-N-benzylaniline, Antergan

Antergan, a synthetic antihistamine, usually sold as the hydrochloride, is estimated by the green color of its nitroso derivative with nitrosoresorcinol.[112] The colored product is probably indaniline. Only slightly more than 1 ppm. is needed for detection and over the preferred range accuracy to ±10 per cent is readily attained.

Samples—*Urine. Total Antergan.* Use directly, adding 1 drop of concentrated hydrochloric acid per 5 ml.

Free antergan. Add 2 ml. of 20 per cent sodium carbonate solution to a 50-ml. sample. Extract with two successive 25-ml. portions of ether. Wash the combined ether extracts with 5 ml. of 0.04 per cent sodium hydroxide solution. Extract the Antergan from the ether with 10 ml. of 1:120 hydrochloric acid and use suitable aliquots of this extract.

Blood. Total Antergan. Mix 5 ml. of blood with 35 ml. of 0.05 per cent saponin solution and after 5 minutes add 10 ml. of 15 per cent trichloroacetic acid. Mix and, after 5 minutes, filter.

Free Antergan. To an aliquot of a trichloroacetic acid filtrate from unlaked blood add solid sodium bicarbonate until there is no further reaction and excess is present. Complete as for urine, starting at "Extract with two successive 25-ml. portions . . . ."

Milk. Treat as for blood.

Procedure—The sample should be in 1:120 hydrochloric acid or 3 per cent trichloroacetic acid. Dilute a volume containing 0.05-1 mg.

[112] P. Dubost, *Ann. pharm. franc.* **1**, 145-8 (1943); G. Biozzi and E. Malizia, *Boll. soc. ital. biol. sper.* **24**, 732-4 (1948).

of Antergan to 5 ml. with the same menstruum. Add 2 drops of 1 per cent aqueous resorcinol solution and mix well. Add 0.5 ml. of 5 per cent sodium nitrite solution and mix. Read after 15 minutes against a reagent blank. Samples derived from urine have an interfering yellow which modifies the green.

α-Diethylamino-2,6-acetoxylidene, Xylocaine

Xylocaine is determined as the reineckate with acuracy to ±2 per cent.[113]

Procedure—Dilute a sample containing about 25 mg. of xylocaine to 10 ml. with water. Add 1:1 hydrochloric acid to adjust to pH 2. Add 5 ml. of saturated aqueous solution of ammonium reineckate dropwise and, after about 10 minutes, filter. Wash the precipitate with 2 ml. and 2 ml. of water. Dissolve the precipitate in acetone and dilute to 50 ml. with that solvent. Read at 530 mμ against a reagent blank.

10-(2-Dimethylaminopropyl)phenothiazine

Follow the procedure for xylocaine.

Diethylamine-*p*-aminophenylstibinate

Diethylamino-*p*-aminophenylstibinate at 0.1 mg. or more per ml. is diazotized and coupled with α-naphthol for estimation from the red color obtained.[114] Urea stibamine interferes in about 10 times the above concentration.

Procedure—To 0.5 ml. of sample add 1 drop of 1:1 hydrochloric acid and 1.5 ml. of water. Cool in ice for 10 minutes and add a drop of a 1 per cent solution of sodium nitrite. Mix and again cool in ice for 1 minute. Add 1 ml. of a 1 per cent solution of α-naphthol in 20 per cent sodium hydroxide solution and mix. Let the color develop for 5 minutes and read against a reagent blank.

Diphenylamine

The familiar oxidation-reduction indicator property of diphenylamine as diphenylamine violet is used for its estimation.[115] The method

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113 Bertil Örtenblad and Karin Jonsson, *Acta Chem. Scand.* **5**, 510–18 (1951).

114 T. C. Boyd and A. C. Roy, *Ind. Med. Gaz.* **64**, 382 (1929).

115 A. Thiel, *Z. Elektrochem.* **35**, 274–8 (1929); H. Barnes, *Analyst* **69**, 344 (1944).

is accurate to ±1 per cent. Ferric sulfate or potassium dichromate are appropriate oxidizing agents which do not introduce sufficient color to interfere. Diphenylamine also develops a color with Nessler's reagent having a maximum below 400 mμ.[116] An alternative is to react with diazo sulfanilic acid in acid solution to give a violet-red.[117] Diphenylamine in ethanol is read in the ultraviolet.[118]

Sample—*D & C Yellow No. 1 or No. 2.* Extract 2.5 grams of dye in a Soxhlet with ether for 4 hours. Extract the ether extract with water made slightly alkaline. Evaporate the ether nearly to dryness, take up in ethanol, and dilute to 100 ml. with ethanol for reading in the ultraviolet.

Procedure—*Oxidation by ferric ion.* Dilute the sample with concentrated sulfuric acid and water to give a final concentration of 1:1 sulfuric acid and 0.004-0.4 mg. of diphenylamine per ml. To 1 ml. of sample add 9 ml. of saturated ferric sulfate solution in 50 per cent sulfuric acid. Let stand for 30 minutes or heat in boiling water for 5 minutes and cool. Read against a reagent blank.

Oxidation by dichromate. Add to 21 ml. of neutral sample containing diphenylamine, 0.2 ml. of 0.04 per cent aqueous potassium dichromate. Cool and add concentrated sulfuric acid with cooling until the volume of the cold solution is 30 ml. Read against a reagent blank.

In the ultraviolet. Read the sample and a standard at 255 mμ, 285 mμ, and 315 mμ. With A_u = values from the unknown and A_s those from the standard calculate

$$X = \frac{A_u\,285\ \text{m}\mu - [(A_u\,225\ \text{m}\mu + A_u\,315\ \text{m}\mu)/2]}{A_s\,285\ \text{m}\mu - [(A_s\,225\ \text{m}\mu + A_s\,315\ \text{m}\mu)/2]}$$

Toluidines

The method of diazotizing and coupling aniline with Chicago acid is applicable with simple modifications to toluidines. In the absence of aniline the method with hypochlorite can be applied to estimation of

[116] Herman A. Liebhafsky and Lester B. Bronk, *Anal. Chem.* **20**, 588-9 (1948); For details see phenyl-α-naphthylamine (page 236).

[117] B. V. Ponomarenko, *Zavodskaya Lab.* **13**, 937-41 (1947).

[118] John E. Clements and Lee S. Harrow, *J. Assocn. Official Agr. Chemists* **35**, 159-61 (1952).

o-toluidine, the oxidized sample being made alkaline.[119] The method will detect 0.25 ppm.

Sample—*Toluidines in methyl or ethyl toluidines.* Dissolve 1 ml. of sample in 1:50 hydrochloric acid and dilute to 100 ml. with that acid.

Yellow AB and Yellow OB. Prepare the sample as described under aniline (page 198) and develop with hypochlorite.

Procedure—*M-Toluidine in methyl toluidines by Chicago acid.* Proceed as for aniline (page 198) through ". . . to decompose excess nitrite." Add 2 ml. of 1 per cent solution of Chicago acid in 1:50 hydrochloric acid to the sample. Add 10 ml. of 50 per cent sodium acetate solution to it. Heat sample and blank on the steam bath for 5 minutes and cool. Add Chicago acid reagent and sodium acetate to the blank, comparable to those added to the sample. Add 50 ml. of methanol to each, dilute to volume with water, and read.

o-Toluidine in ethyl toluidines by Chicago acid. Transfer 1 ml. of sample solution to a 100-ml. flask. As blank start with an empty flask at this point. Proceed as for aniline (page 198) through ". . . to decompose all excess nitrite." Add 5 ml. of 1 per cent solution of Chicago acid in 1:50 hydrochloric acid to each. Add 10 ml. of 50 per cent sodium acetate solution to each. Warm both to 30°, cool, add water to dilute to volume, and read.

o-Toluidine with hypochlorite. Follow the procedure under aniline (page 199).

Diethyl-m-toluidine

The reaction as the *p*-nitroso compound described for tertiary amines derived from aniline is applicable. The method is not applicable to diethyl-*o*-toluidine and diethyl-*α*-naphthylamine because their nitroso compounds have too low tinctorial power.

Sample—*Diethyl-m-toluidine in m-toluidine and/or monoethyl-m-toluidine.* Dissolve 1 ml. in 1:50 hydrochloric acid and dilute to 100 ml. with the same acid.

Procedure—Transfer 2 ml. of sample solution, 1 ml. of glacial acetic acid, 50 ml. of water, and 5 ml. of saturated sodium nitrite solution. After 1 minute add 25 ml. of methanol and dilute to 100 ml. with water.

119 O. L. Evenson, J. A. Kime, and S. S. Forrest, *Ind. Eng. Chem., Anal. Ed.* **9**, 74-5 (1937).

Mix and let stand for 10 minutes. Complete as for dialkylanilines (page 200), starting at "Stopper, mix by inversion . . . ."

p-Phenylenediamine

In substantially neutral aqueous solution, p-phenylenediamine is used to reduce phosphotungstic acid to give a blue color.[120] The reagent will form a red precipitate if added to an acid solution around pH 5, which will not redissolve at pH 8. After adjusting the solution to pH 7, on addition the reagent develops a purple color and a clear blue is obtained when this is raised to pH 8. The same color is given by substituted p-phenylenediamines, p-aminophenol, hydroquinone, catechol, pyrogallol, α-naphthol, ferrous chloride, and thiosulfate. There is no reaction with phenol, cresol, aniline, β-naphthol, thymol, and salicylic acid. Both the intensity and stability of the color are affected by pH. There is interference by wood-tar distillates but not by dyes or tetraethyl lead.

The blue color of an aqueous solution of p-phenylenediamine with aniline and ferric chloride can be used for colorimetric estimation. Other phenylene and tolylene diamines, aminophenols, and related compounds give similar but not identical colors. Under suitable conditions 0.001 mg. can be detected.[121] A method of determination in the presence of 2,5-diaminotoluene is given under the latter topic (page 229).

Sample—*Gasoline.* Extract 100 ml. with 20 ml. of 1:4 hydrochloric acid by mechanical shaking for 3 minutes. Re-extract with 10 ml. of the acid for 1 minute. Wash the gasoline with 20 ml. of distilled water and combine the extracts. If colorless, they are ready for development. If colored, extract with 20-ml. portions of 1:1 toluene and isooctane until all dye has been removed. Dilute to a known volume. Develop an aliquot with phosphotungstic acid reagent. This sample is also used for p-aminophenol.

Procedure—*By phosphotungstic acid.* As reagent reflux 100 grams of sodium tungstate, 750 ml. of distilled water, and 80 ml. of 85 per cent phosphoric acid for 2 hours. Cool, filter, and dilute to 1 liter. All

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[120] Lois R. Williams and Barney R. Strickland. *Anal. Chem.* **19**. 633-4 (1947).

[121] A. S. Zhitkova, S. I. Kaplun and Joseph B. Ficklen, "Estimation of Poisonous Gases and Vapors in the Air," pp. 171-2. Service to Industry, West Hartford, Conn. (1936).

reagents must be nitrate-free. To an appropriate aliquot of sample add 18 per cent aqueous sodium carbonate with stirring until pH 7 is reached with a glass electrode. Add 2.5 ml. of phosphotungstic acid reagent and continue to add sodium carbonate until pH 8 is reached. Dilute to 50 ml., mix, and read at 650 mμ.

By aniline and ferric chloride. Transfer a neutral sample solution containing 0.05-0.5 mg. of *p*-phenylenediamine and dilute to about 40 ml. Add 5 ml. of a 1 per cent aniline solution and mix. Add 1 ml. of 1 per cent ferric chloride hexahydrate solution and mix. Let stand for 30 minutes and read against a reagent blank.

N,N^1-Diethyl-*p*-phenylenediamine

The color reaction with chloramine T and phenol is applicable to this compound.[122]

Procedure—Treat 5 ml. of an aqueous solution with 0.1 ml. of 5 per cent phenol solution, 0.1 ml. of 10 per cent sodium carbonate solution, and 0.1 ml. of 1 per cent chloramine T solution. Let stand for 20 minutes and read against a reagent blank.

p-Aminoacetanilide

p-Aminoacetanilide is read directly in the ultraviolet.[123] The results are accurate to ±1 per cent.

Sample—*D & C Red No. 1.* Mix 0.5 gram of sample with 50 ml. of ether. Filter, and rinse the residue and filter with 10 ml. of ether. Resuspend the residue and filter paper in 50 ml. of ether. Filter and wash the residue and paper with 10 and 10 ml. of ether. Extract the combined ether solutions with five 20-ml. portions of 1:99 hydrochloric acid. Heat the combined acid extracts on the steam bath for a half-hour to vaporize dissolved ether. Cool and dilute to 100 ml. with 1:99 hydrochloric acid.

Procedure—Read at 241 mμ against 1:99 hydrochloric acid.

[122] T. V. Solovéva, *Gigiena i. Sanit.* 1953, No. 1, 47-8.

[123] K. S. Heine, Jr., and Wm. J. Sheppard, *J. Assoc. Official Agr. Chem.* 34, 802-9 (1951).

2,5-DIAMINOTOLUENE

p-Phenylenediamine and 2,5-diaminotoluene are frequently used in hair dyes, either singly or together.[124] Gravimetric methods for determining each of these diamines are not applicable when both are present.[125] Mixtures of these diamines are determined as their diacetyl derivatives spectrophotometrically. Since solutions of the free diamines are not stable, alcoholic solutions of the diacetyl derivatives are used. There is no interference from the polyhydric phenols which are contained in many commercial hair dyes.

Procedure—Dissolve the sample in 6 ml. of 1:5 hydrochloric acid and transfer to a separatory funnel with 15 ml. of water. Extract with five 20-ml. portions of ether discarding the extracts. Transfer the remaining solution to a continuous extractor and add 10 ml. of 50 per cent sodium hydroxide and 5 ml. of 1 per cent aqueous sodium sulfite. Extract for 4 hours with ether. Extract with a fresh 15-ml. portion of ether to test for complete extraction. The residue on evaporation of this portion should be negligible. Filter the extract through a cotton plug into 2 ml. of acetic anhydride. Wash the extraction flask and cotton plug several times with ether and then evaporate off the solvent on the steam bath. Dry the residue at 100° to constant weight.

Dissolve the residue in a measured amount of ethanol so that there is a final concentration of about 5 mg. per liter. Read at 250 and 270 mμ. Calculate as follows:

$$D_{250} = xd_p^{250} + yd_t^{250}$$
$$D_{270} = xd_p^{270} + yd_t^{270}$$

where x = concentration of diacetyl *p*-phenylenediamine
y = concentration of diacetyl-2,5-diaminotoluene
D_{250} and D_{270} are the optical densities of the unknown at 250 and 270 mμ.
d_p^{250} *and* d_p^{270} are optical densities per unit concentration of diacetyl-*p*-phenylenediamine at the respective wave lengths
d_t^{250} and d_t^{270} are similar values for diacetyl diaminotoluene

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124 S. H. Newburger and J. H. Jones, *Ibid.* 33, 374-9 (1950).

125 C. Griebel and F. Weiss, *Z. Unters. Lebensm.* 65, 419 (1933); *Ibid.* 67. 86 (1934); R. Viollier and J. Studinger, *Mitt. Lebensm. Hyg.* 24, 194 (1933).

4,4'-Diaminobiphenyl, Benzidine

Benzidine in aqueous alkaline solution reacts quantitatively with sodium-β-naphthoquinone-4-sulfonate to give an appropriate blue color.[126]

Sample—*Benzidine sulfate.* Dissolve the sample in a solution of 1 per cent sodium borate and 0.4 per cent sodium hydroxide. Heat in boiling water for 10 minutes and dilute to a known volume.

Procedure—Mix 2 ml. of alkaline sample solution with 1 ml. of 0.15 per cent sodium-β-naphthoquinone-4-sulfonate solution. After 5 minutes add 2 ml. of acetone to reduce the color of excess reagent. Read at 500 mμ against a reagent blank.

2,4'-Diaminobiphenyl, Diphenyline

Diphenyline, when tetrazotized and coupled with 1-amino-8-hydroxy-2,4-naphthalene disulfonic acid, Chicago acid or SS acid, gives a red azo dye in the presence of bicarbonate.[127] Accuracy to ±5 per cent is obtainable. The usual occasion to determine it is an impurity in benzidine, 4,4'-diaminobiphenyl. Since benzidine gives somewhat analogous colors, the major portion must be separated.

Sample—*Benzidine.* Dissolve a 2-gram sample, adding 1:10 hydrochloric acid if the sample is as free base. Add 25 ml. of excess acid and dilute to 150 ml. Heat to dissolve, cool, and dilute to 250 ml. If turbid, filter through a dry filter. Slowly add a 25-ml. aliquot, with constant stirring, to 25 ml. of 1:2 sulfuric acid heated to boiling. During precipitation, keep the solution boiling moderately. Continue to heat with stirring for about 5 minutes. Cool with a mixture of ice and salt to about –15° C. and maintain this temperature for 20 minutes. Moisten a filter with 1:5 sulfuric acid cooled to –15° C.

During filtration, which takes about 0.5 hour, the temperature of the solution on the filter must not exceed –6° C. Use an insulated filter such as the usual device for hot filtration. Lift the cover as briefly as possible. Under these conditions, there is a constant concentration of benzidine in the filtrate.

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[126] T. V. Letonoff and John G. Reinhold, *J. Biol. Chem.* **114**, 147-56 (1936); Oscar Touster, *Ibid.* **188**, 371-7 (1951).

[127] V. Cech and K. Kámen, *Chem. Listy* **33**, 97-101 (1939).

Wash with about 30 ml. of 1:5 sulfuric acid cooled to −15° C. Collect 75-80 ml. of filtrate and washings and carefully add 20 grams of solid anhydrous sodium carbonate to neutralize most of the acid. Close the flask with a small funnel to prevent loss. Cool to 0° C., add 4 ml. of 18 per cent sodium nitrite solution, stir, and after 2 minutes remove excess nitrous acid by gradually introducing 10 grams of solid urea in small amounts. Leave in ice for 5-10 minutes with occasional stirring until the reaction with starch iodide paper is negative. Dilute to 500 ml. with ice-cold water. The solution is stable for 12 hours if kept on ice.

If the solution contains less than 0.1 per cent of diphenyline, prepare a reagent blank. For this dissolve 0.07 gram of pure benzidine in a small volume of water and 5 ml. of 1:10 hydrochloric acid. Dilute to 1 liter and transfer 1 ml. of this. Add a little water, then 10 ml. of 1:5 sulfuric acid, cool to 0°, and add 2 ml. of 18 per cent sodium nitrite solution. After 2 minutes, add 10 grams of urea. When reaction with starch-iodide paper disappears, dilute to 1 liter with ice water. Keep cooled with ice.

Procedure—To 5 ml. of 8.4 per cent sodium bicarbonate solution add 5 drops of 1 per cent SS acid solution. Add 5 ml. of prepared sample with stirring. Dilute to 15 ml. with water and read against the reagent blank, with or without benzidine present.

Aromatic Amidines

After coupling with glyoxal, aromatic amidines yield colored derivatives when warmed with alkali or condensed with aldehydes in alkaline solution. The red pigment formed is a glyoxaline. The method has been applied colorimetrically [128] to *p*-methylsulfonylbenzamidine—known commercially as V 187—4,4′-diamidinodiphenoxypropane or propamidine, and 4,4′-diamidinostilbene or stilbamidine; and fluorimetrically [129] to stilbamidine, propamidine, pentamidine, phenamidine, and *p*-carbethoxybenzamidine. About 0.01-0.5 mg. of amidine can be determined colorimetrically, 0.0005-0.01 mg. fluorimetrically, each with an average error of ±6 per cent.

[128] J. Devine, *Ann. Trop. Med. Parasit.* **38**, 35-45 (1944); A. T. Fuller, *Nature* **154**, 773 (1944); *Biochem. J.* **39**, 99-102 (1945); C. W. Ballard, *Quart. J. Pharm.* 21. 376-87 (1948).

[129] A. T. Fuller, *Biochem. J.* **39**, 99-102 (1945); Dudley P. Jackson, W. James Kuhl, and J. Logan Irvin, *J. Biol. Chem.* **167**, 377-86 (1947).

The reaction is specific for an unsubstituted amidine directly joined to an aromatic nucleus. It is not given by an aromatic amidine with one or two methyl groups on the nitrogen atoms of the amidine group, or by benzamidrazone, phenyl acetamidine, guanidines, biguanides, or aliphatic amidines.

Since amidines are precipitated by the usual protein coagulants, colloidal iron is used for protein precipitation, or, to determine smaller amounts, the amidine is extracted with isopropanol or butanol. To prevent precipitation of the pigment when determined colorimetrically, a protective colloid is added. The fluorimetric reading is taken in an aqueous ethanol-ether solution. The color varies with the concentration of reagent and is at a maximum at 1-2 mols of glyoxal per mol of amidine. It also varies with the pH; color is developed at pH 9.6 with use of a borate buffer solution.

Samples—*Blood.* For colorimetric development mix 2 ml. of blood with 8 ml. of isopropanol and filter to remove proteins.

Plasma. For fluorimetric development, add to 5 ml. of plasma 15 ml. of water and immerse the container in boiling water for 3-4 minutes or until turbidity appears. Add dropwise with constant agitation 3 ml. of dialyzed iron equivalent to a 5 per cent suspension of ferric oxide. Heat for 2 minutes and add dropwise with agitation 2 ml. of a 22 per cent solution of anhydrous sodium sulfate. Cool to room temperature and centrifuge for 5-10 minutes. Use the centrifugate as sample and for preparation of a blank. This should be colorless but may be slightly turbid.

Alternatively, to extract the amidine with butyl alcohol, add 25 ml. of *n*-butanol to 5 ml. of plasma in a centrifuge tube. Stopper, shake for 2 minutes, and centrifuge. Decant the centrifugate and extract a second time with 25 ml. of butanol. Combine the extracts and acidify to Congo red with 1:5 sulfuric acid. Distil off the butanol *in vacuo* at 40-60°. Dissolve the residue in 12 ml. of water and extract lipides and bilirubin with 10 ml. of chloroform. Use the aqueous solution as sample.

Since the sample will yield some fluorescence, prepare a blank with 10 ml. of the centrifugate of plasma from which proteins were removed with dialyzed iron. Add to this 1 ml. of 40 per cent sodium hydroxide solution and immerse in a boiling-water bath for 15 minutes in order to hydrolyze the amidine present. Cover the mouth of the flask with a small funnel to minimize evaporation during hydrolysis. Cool and treat

the same as the sample except for addition of sodium hydroxide after the reagents have been added.

Urine. For colorimetric development use urine undiluted or, according to concentration, at any dilution necessary down to 1:100.

For fluorimetric development, dilute 5 ml. or less of urine containing 0.002-0.01 mg. of amidine to 10 ml. and use as sample. If necessary, make a preliminary analysis. If the amidine content is low, do not use more than 10 ml. of urine, since it contains inhibitory substances and pigments. If 10 ml. of urine is taken, increase the volumes of reagents and alkali by 50 per cent.

Pharmaceutical preparations. As a sample for colorimetric development, prepare an aqueous solution of an opthalmic liquid containing amidine, of an injection liquid, or of a jelly, to contain about 0.1 mg. of amidine per ml.

Procedure—*Colorimetric.* To 5 ml. of sample solution add 2 ml. of a 2 per cent solution of glyoxal sodium bisulfite, 10 ml. of a borate buffer solution containing 2.76 grams of boric acid and 23.4 ml. of 0.4 per cent sodium hydroxide solution per 100 ml., and 5 ml. of a 1 per cent gelatine solution as protective colloid. Mix the treated sample, heat in boiling water for 10 minutes, and cool in ice water for 5 minutes. Dilute to 25 ml. and read at about 520 mμ, with heat-absorbing filter H 503. Correct for the value of a blank treated the same except that heating is omitted. Alternatively compare with a similarly treated standard containing 0.1 mg. per ml. of the aromatic amidine being detemined.

Fluorimetric. Plasma. To 10 ml. of sample solution add 0.4 ml. of 2 per cent solution of glyoxal sodium bisulfite and 0.5 ml. of an 8 per cent solution of benzaldehyde in ethanol. Mix and add dropwise with constant agitation 1 ml. of 40 per cent sodium hydroxide solution. Add 10 ml. of a 1:9 ether-ethanol mixture. Shake mechanically for 45 minutes. After 15 minutes measure the fluorescence by comparison with a standard calibration curve or, preferably, with 10 ml. of a similarly treated standard solution containing 0.001 mg. of the proper amidine per ml.

If the fluorescent solution is turbid, filter through sintered glass, using gentle suction to avoid evaporation of alcohol and ether. Allow for the fluorescence of the blank. In making the reading use as primary filter one having a mean transmission at 410 mμ, and a secondary filter transmitting radiation of wave length greater than 490 mμ. These

correspond to Corning Filters 5113 and 3385 respectively, or Coleman No. B_2 and PC-9, respectively.

Urine. Mix 10 ml. of sample, 0.8 ml. of 2 per cent glyoxal sodium bisulfite, and 1 ml. of 8 per cent benzaldehyde in ethanol. Add 2 ml. of 40 per cent sodium hydroxide solution with agitation and shake mechanically for 45 minutes. Extract the solution with 20 ml. of *n*-butanol, centrifuge, and discard the aqueous layer. Wash the butanol extract by shaking with 10 ml. of 0.4 per cent sodium hydroxide solution. Centrifuge and discard the aqueous wash liquid. To 15 ml. of butanol extract add 5 ml. of ethyl ether and filter after 15 minutes. Measure the fluorescence of the filtrate, using the filters specified for the plasma sample. Deduct a blank determination obtained on the same volume of urine as the sample, diluted to 10 ml. and boiled with 2 ml. of 40 per cent sodium hydroxide solution for 15 minutes prior to development of fluorescence.

p,p'-(Trimethylenedioxy)dibenzamidine bis(β-hydroxyethanesulfonate), Propamidine

The reaction of propamidine with fresh pentacyanoammonioferrate is suitable for reading.[130]

Procedure—*Serum.* Mix 2.5 ml. with 7 ml. of water and heat to boiling. Add 2 ml. of 5 per cent dialyzed iron, equivalent to a 5 per cent suspension of ferric oxide, dropwise with swirling, and heat for a couple of minutes. The solution should settle clear. Mix 2 ml. of the supernatant liquid with 1 ml. of 0.1 per cent pentacyanoammonioferrate, freshly prepared by dilution. Read against water after a half-hour.

Dibromopropamidine

The procedure for propamidine is also applicable to this compound.

α-Naphthylamine

The reaction by diazotizing and coupling with Chicago acid as applied to aniline is applicable with rather simple modifications to α-naphthylamine. It is also coupled with sodium benzenesulfonate

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[130] Hilda Trought, G. C. Aston, and R. G. Baker, *Analyst* 75, 437-40 (1950).

p-diazonium chloride, diazotized p-phenylenediamine,[131] or with β-naphthol.[132]

Samples—*α-Naphthylamine in ethyl naphthalenes.* Dissolve 1 ml. in 1:50 hydrochloric acid and dilute to 100 ml. for the development of aliquots with Chicago acid.

General. Dilute to a content of α-naphthylamine expected to approximate 0.005 mg. per ml.

Air. Draw the air through a cotton filter moistened with 1:10 hydrochloric acid. When absorption is of the order of 0.1-0.25 mg., extract the cotton with 5 ml. of the acid and 25-30 ml. of water as a sample for development with β-naphthol.

Procedure—*By Chicago acid.* Proceed as for aniline (page 198) through ". . . to decompose all excess nitrite." To one flask add 5 ml. of 1 per cent Chicago acid in 1:50 hydrochloric acid. To each add 10 ml. of 50 per cent sodium acetate solution. Remove the sample flask from the ice bath and heat to 30° by immersion in warm water. Do not warm the blank. Cool the sample, add 50 ml. of acetone to each, dilute to volume with water, and mix. Read the sample against the blank.

By sodium benzenesulfonate p-*diazonium chloride.* As reagent dissolve 0.27 gram of p-aminobenzene sodium sulfonate dihydrate in 25 ml. of water. Render distinctly acid with 1:1 hydrochloric acid, and cool. Add 0.7 per cent solution of sodium nitrite until the reaction is almost complete but stop just short of the end point, as shown by starch-iodide paper, to avoid the presence of any excess nitrous acid. Dilute to 100 ml. Mix 1 ml. of sample with 0.1 ml. of glacial acetic acid and dilute to 10 ml. with the reagent. Let stand for 2-3 minutes and read against a reagent blank.

By diazotized p-*phenylenediamine.* As reagent dissolve 0.38 gram of p-phenylenediamine in 40 ml. of water and 1 ml. of concentrated hydrochloric acid. Chill with ice and add 0.7 per cent sodium nitrite solution nearly to the starch-iodide end point. Dilute to 500 ml. Mix 10 ml. of sample and 30 ml. of 30 per cent acetic acid, and add 3 ml. of the reagent. After about 5 minutes read against a reagent blank.

By β-naphthol. Dilute the sample containing 10 ml. of 1:10 hydro-

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131 V. Lenkhold, *Anilinokrasochnaya Prom.* **3**, 87-8 (1933).

132 B. V. Ponomarenko, *J. Applied Chem.* (USSR) **11**, 147-50 (1938).

chloric acid to about 35-40 ml. Chill in ice and add 3 ml. of 10 per cent sodium nitrite solution. After 15 minutes neutralize by addition of 20 per cent sodium carbonate solution. Add a drop of 4 per cent solution of β-naphthol in ethanol and dilute to 50 ml. After 15 minutes read against a reagent blank.

β-Naphthylamine

The color of β-naphthylamine in alkaline solution is used for analysis of certifiable colors for β-naphthylamine content.[133] On oxidation with hypochlorite in acid solution β-naphthylamine gives a yellow color, whereas the most probable analogous amines, aniline and o-toluidine, give the color only in alkaline solution.[134] The method will determine 0.25 ppm.

Sample—*Yellow AB and Yellow OB.* The preparation of sample for development with hypochlorite is described under aniline (page 198).

Procedure—*Alkaline development. FD&C Yellow 3 or 4.* Add to a 500-ml. flask in order a few boiling chips, 80 grams of sodium chloride, 5 grams of sample, wet glass wool to fill three-quarters of the flask, 5 ml. of 30 per cent sodium hydroxide solution, and 100 ml. of water. Distil at 2 drops a minute through a steam trap into 5 ml. of 1:1 hydrochloric acid. After collecting 125 ml., add 100 ml. of water to the distilling flask and distil to collection of 200-225 ml. Make the distillate alkaline to litmus with 30 per cent sodium hydroxide. Extract the distillate and washings of the receiver with three 50-ml. portions of ether. Extract the combined ether solutions with four 10-ml. portions of 1:60 hydrochloric acid. Heat the acid extracts on a steam bath to drive off ether, cool, and add 45 ml. of 1.6 per cent sodium hydroxide solution. Dilute to 100 ml. and read at 305, 335, or 354 mμ. against a reagent blank.

Oxidation with hypochlorite. Follow the technic under aniline (page 199).

Phenyl-α-naphthylamine

Nessler's reagent, conventionally used for estimation of ammonia, gives a more intense color with an equivalent amount of phenyl-

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133 Lee S. Harrow, *J. Assoc. Official Agr. Chem.* **34**, 131-2 (1951).

134 O. L. Evenson, J. A. Kime, and S. S. Forrest, *Ind. Eng. Chem., Anal. Ed.*, 9, 74-5 (1937).

α-naphthylamine.[135] The color continues to develop for at least **16** hours and has a maximum below 400 mμ. Aniline, diamylamine, propylenediamine, and triethylamine do not develop a color, but diphenylamine does.

Procedure—Dilute a sample containing 0.005-0.01 mg. of nitrogen as phenyl-α-naphthylamine to about 22 ml. Add 1 ml. of Nessler's reagent (page 181) and dilute to 25 ml. Read after a definite time, such as after 16 hours, at a specific wave length as at 400 mμ. If cloudiness has developed, centrifuge.

2-Aminofluorene

2-Aminofluorene when diazotized and coupled with sodium 2-naphthol-3,6-disulfonate, the sodium salt of R acid, gives a pink to orange color almost instantly.[136] Results are reproducible to ±5 per cent. A port-wine color of the amine with sodium 1,2-naphthoquinone-4-sulfonate, is also applicable.

Sample—*Tissue.* Mince a sample expected to contain about 0.3 mg. and extract with 9 parts of acetone, carefully measured as to volume. Centrifuge and pipet off half the volume added. Evaporate to dryness at not over 65° and take up the residue in 0.5 ml. of glacial acetic acid for development.

Aqueous solutions. Dilute to about 0.2 mg. per ml. for development.

Procedure—To 1 ml. of aqueous sample add 0.5 ml. of glacial acetic acid, or to 0.5 ml. of sample in glacial acetic acid add 1 ml. of water. This should contain 0.03-0.2 mg. of 2-aminofluorene. Mix and add 1.2 ml. of 0.2 per cent sodium nitrite solution. After a minute pour into 10 ml. of 5 per cent sodium 2-naphthol-3,6-disulfonate in 1:3 ammonium hydroxide. Read against a reagent blank at 525 mμ.

N-Acetyl-2-aminofluorene

Saponify and determine as 2-aminofluorene.

N,N-Diacetyl-2-aminofluorene

Saponify and determine as 2-aminofluorene.

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135 Herman A. Liebhafsky and Lester B. Bronk, *Anal. Chem.* **20**, 588-9 (1948).

136 Benton R. Westfall, *J. Natl. Cancer Inst.* **6**, 23-9 (1945); Benton R. Westfall and Harold P. Morris, *Ibid.* **8**, 17-21 (1947).

CHAPTER 6

AZO COMPOUNDS, NITROGEN-CONTAINING CYCLES, ETC.[1]

The nitrogen compounds grouped in this chapter are inherently more complex than those in the preceding chapters. That does not necessarily mean that this is more complex than any of those preceding. There is no cohesive class. Group reactions include that of the pyridine ring with cyanogen bromide and an amine, imidazoles with diazotized sulfanilic acid, or the antihistamines with reineckates. Many are determined by diazotizing and coupling.

Azobenzene

When absorbed in nitrating acid according to a technic described for nitrobenzene (page11), azobenzene is converted to p,p′-dinitrodiphenyldiimide which is estimated by its violet color with an aldehyde or ketone and sodium hydroxide.[2]

p-Aminoazobenzene

p-Aminoazobenzene is read in acid solution after appropriate isolation.[3] The method is applicable to 0.005 mg. of dye, with an accuracy of ±5 per cent. *p*-Monomethylaminoazobenzene and *p*-dimethylaminoazobenzene interfere but are separable chromatographically. The three can also be read together. Compounds containing acidic groups do not interfere. It is an intermediate in preparation of the certified coal-tar dye, Ext. D and C Red No. 13, determined by its orange color in acid solution after extraction with benzene.[4]

Sample—*Tissue.* Homogenize 4 grams of fresh tissue with 5 ml. of water and transfer with 7 ml. of ethanol. Add 2 ml. of 60 per cent potassium hydroxide solution, mix, and let stand with occasional shaking for 90 minutes. Add 10 ml. of petroleum ether and stir mechan-

[1] See Volume III, Chapter 1, for details of organization, condensation, etc.

[2] P. K. Bose, *Z. anal. Chem.* 87, 110-14 (1932); M. S. Bykhovskaya, *Org. Chem. Ind. (USSR)* 6, 638-9 (1939).

[3] J. A. Miller and C. A. Baumann, *Cancer Research* 5, 157-61 (1945).

[4] S. H. Newburger, *J. Assoc. Official Agr. Chem.* 25, 947-8 (1942).

ically for 5 minutes. If an emulsion forms, add a few drops of ethanol. Extract twice more and combine the extracts. Add 6 ml. of concentrated hydrochloric acid mixed with 4 ml. of water and shake vigorously. This solution contains the mono- and dimethyl-derivatives if present, as well as the aminoazobenzene. It can be read in the ultraviolet for the sum of the three or for any single one present. If more than one is present, they can be separated.

Fractionation. To fractionate the acid solution of the azo dyes, remove the petroleum ether layer containing unsaponifiable matter, immerse in cold water, and add 9 ml. of 60 per cent potassium hydroxide solution slowly, keeping the temperature below 60° C. Extract the alkaline solution with three 10-ml. portions of petroleum ether, as described for the tissue hydrolyzate.

Add a few boiling chips and remove the solvent from the extract *in vacuo* at 50-60°. Wash down successively with four 1-ml. portions of petroleum ether and transfer quantitatively to an alumina sorption column 12 cm. long and 4 cm. in diameter, tapered at the lower end. To prepare the column use a very weakly sorbent 100-mesh alumina. Moisten with methanol and dry at 90-100°. Screening or sedimentation may be necessary to remove very fine particles which would clog the column.

After the petroleum ether solution passes through the column, wash with 0.5 ml. of petroleum ether, 1 ml. of a 1:1 mixture of petroleum ether and benzene, then with pure benzene. As the amount of benzene in the wash solution is increased, three bands form and travel down the column.

As the bands approach the end of the column, each band is eluted until the next is within a few mm. of the end of the column. Elute each band into a separate tube and wash down the tubes and the tip of the column with 10 ml. of petroleum ether. Add 10 ml. of 7:3 hydrochloric acid to each and read.

D and C Red No. 13. Dissolve 1 gram of dye in 250 ml. of water and extract with 50, 30, and 30 ml. of benzene. Combine the benzene extracts and wash first with 50-ml. portions of 0.4 per cent sodium hydroxide solution until the washings are colorless and then with two 25-ml. portions of water. Filter the benzene solution through cotton, add 30 ml. of water to the filtrate, and heat on the steam bath until the benzene is evaporated. Add 50 ml. of ethanol, stir to dissolve, and dilute to 100 ml. with water for reading.

Procedure—Read at 510 mμ for the combined azo dyes, or at 500 mμ for *p*-aminoazobenzene, 505 mμ for *p*-monomethylaminoazobenzene, and 518 mμ for dimethylaminoazobenzene.

p-Methylaminoazobenzene

This is separated and isolated as described under *p*-aminoazobenzene.

p-Dimethylaminoazobenzene

This is separated and isolated as described under *p*-aminoazobenzene.

Aminoazoxylene

Aminoazoxyene—used as an intermediate in the preparation of certified coal-tar dyes—is determined in D and C Red No. 18, Oil Red OS, 1-xylylazo-2-naphthol, after extraction from petroleum ether with a dilute acidified ethanol solution, and steam distillation.[5] Recoveries average about 90 per cent on samples containing known quantities of the intermediate. Xylidines, if present, distil with steam, but do not interfere with the determination.

Sample—Dissolve 1 gram of D and C Red No. 18 in 100 ml. of chloroform. Dissolve 5 ml. of this solution in 250 ml. of petroleum ether boiling at 30-65°. Prepare an extraction solution by mixing 4 volumes of 1:4 sulfuric acid with 1 volume of ethanol. Extract the aminoazoxylene with four or more 20-ml. portions of this extraction solution.

Dilute to 100 ml. or to 250 ml. to give a solution containing 0.1 per cent or more of intermediate. Transfer an aliquot containing approximately 0.1 mg. of aminoazoxylene to a steam distillation flask. Cool in ice and make just alkaline to litmus with small quantities of sodium carbonate. Dilute to about 50 ml. and add about 20 grams of sodium chloride. Connect for steam distillation and distil slowly until about 85 ml. of distillate have been collected. Disconnect, wash the condenser with about 15 ml. of ethanol, and add to the distillate.

Extract with 30, 25, and 20-ml. portions of ether. Remove the solvent by sulfuric acid-washed air. Dissolve the residue in 20 ml. of ethanol for use as sample.

Procedure—Compare with a series of standards containing 0.05-0.15 mg. of aminoazoxylene in 20 ml. of ethanol.

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[5] O. L. Evenson, *J. Assoc. Official Agr. Chem.* 27, 572-3 (1944).

Pyrrole

A hydrochloric acid solution of pyrrole gives a red color with *p*-dimethylaminobenzaldehyde.[6] It is conveniently developed by adding the reagent in alcoholic solution and heating to boiling. Beer's law applies up to 70 mg. of pyrrole per liter in 0.2 per cent hydrochloric acid solution. By raising the hydrochloric acid concentration to 4.4 per cent, the color is trebled. Pyridine and pyrrolidine give no color. Pyrrole condenses with isatin in hydrochloric acid solution to give a blue color suitable for reading. With 1:10 hydrochloric acid the color is precipitated if more than 40 mg. of pyrrole per liter is present. At lower concentrations of hydrochloric acid the color is less intense. Pyridine, pyrroline, and pyrrolidine do not give color under these conditions.

The pyrrole group is undesirable in hydrocarbons as promoting gum formation, sedimentation, and discoloration. The compounds are satisfactorily determined with *p*-aminobenzaldehyde.[7] The extraction should be with phosphoric acid-acetic acid. If phenolic compounds are present, they must be washed out with dilute sodium hydroxide solution. The spectra developed from the pyrroles from fuel oil is like that of 2-methylpyrrole which is therefore used as the standard. The maximum absorption at 570 mμ does not conform to Beer's law as well as that used at 540 mμ. Results on cracked gasolines are erratic. Tetrasubstituted pyrroles such as carbazole and tetramethylpyrrole do not react.

Sample—*General.* Measure a volume to contain 0.1-9 mg. of pyrrole. Render it distinctly alkaline with 10 per cent sodium hydroxide solution. Extract 3 times with one-fourth volume of ether and combine the ether extracts. Fit a 1-liter Kjeldahl flask with a 2-holed rubber stopper. Insert a separatory funnel containing the pyrrole in ether in one hole. Insert an air condenser through the other hole. Put 5-10 ml. of glacial acetic acid in the flask. Warm on a water bath at 40-50°. Slowly drop the ether extract of the sample into the flask and warm until the ether is completely driven off. Heating over 50° will polymerize the pyrrole. Dilute the acetic acid solution of pyrrole to 100 ml. with water. If clear, use the sample solution at once to prevent polymerization.

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[6] Fritz Fromm, *Mikrochemie* 17, 141-54 (1935); G. H. Guest and W. D. MacFarlane, *Can. J. Research* 17B, 133-8 (1939).

[7] R. B. Thompson, Ted Symon, and Charles Wankat, *Anal. Chem.* 24, 1465-7 (1952).

If the sample was of biological material, the acetic acid solution of sample at this stage may be turbid. In that case mix a suitable known volume of the sample solution in a centrifuge tube with 2-5 ml. of a saturated aqueous solution of mercuric chloride. After 15 minutes, centrifuge and decant the supernatant liquid. Add 3 ml. of ethanol to the precipitate and warm on a water bath. Cool and centrifuge. Precipitate as before with mercuric chloride solution and centrifuge. Wash the precipitate with water. Carefully warm the precipitate on a water bath and dissolve in the minimum possible volume of 5 per cent sodium cyanide solution. Dilute this solution to 25, 50, or 100 ml. according to the pyrrole content and use as sample. This mercury precipitate must not stand more than a few hours or polymerization will occur. Develop with *p*-dimethylaminobenzaldehyde or isatin.

Coffee infusion. Reflux 10 grams of ground roasted coffee with 100 ml. of boiling water. After 15 minutes, cool, filter, and wash the residue. Render the infusion alkaline with 1 per cent sodium hydroxide solution. Extract the infusion three times with 25-ml. portions of ether. Combine the ether extracts and treat as for the general method.

Coffee grounds. Steam-distil 50 grams of coffee grounds. Over 90 per cent of the pyrrole is present in the first 500 ml. of distillate and substantially all in 1 liter.

Gelatine. Heat 10 grams of air-dried, finely divided gelatine in a retort connected to a condenser, until no further distillate is obtained. Transfer the distillate with the minimum possible volume of ethanol. Acidify with hydrochloric acid and dilute with water. Extract the solution with four 25-ml. portions of ether and proceed with the extracted solution as under the general method.

Procedure—*By p-dimethylaminobenzaldehyde.* To a suitable volume of prepared sample solution add 1 ml. of 1:25 hydrochloric acid. Dilute with water to 10 ml. and add 1 ml. of fresh 1.25 per cent solution of *p*-dimethylaminobenzaldehyde in ethanol. Heat in boiling water for 5 minutes, during which much of the ethanol is driven off. Cool and read at 530 mμ against a sample blank from which the reagent was omitted.

By isatin. Treat a suitable volume of sample solution with 2 ml. of 1:1 hydrochloric acid and dilute with water to 10 ml. Add 1 ml. of 0.05 per cent solution of isatin in glacial acetic acid, mix, and heat in boiling water. Cool after 10 minutes and read at 530 mμ against a reagent blank.

By p-aminobenzaldehyde. Dilute 10 ml. of sample to 50 ml. with low-boiling petroleum ether. Dissolve 0.4 gram of pure *p*-aminobenzaldehyde in 100 ml. of 85 per cent phosphoric acid to give a colorless solution. Add 5 ml. and shake vigorously for 3 minutes. Add 25 ml. of glacial acetic acid and shake for 1 minute. Withdraw the acid layer and wash the residual hydrocarbon with 2.5 ml. of 85 per cent phosphoric acid. Combine this with the acid extract and dilute to 50 ml. with glacial acetic acid. As a reference blank carry 5 ml. of 85 per cent phosphoric acid without reagent through the same procedure. Read the sample at 570 mμ with a PC-4 filter against the blank. The color in the blank darkens rapidly so this should be run as promptly as possible. If color is too intense, dilute the sample extract and blank with acetic acid.

Pyridine

A basic reaction for estimation of the pyridine ring is treatment with cyanogen bromide. Subsequent reaction with an amine develops an intensely colored derivative of glutaconaldehyde.[8] Coupling reagents are aniline,[9] with sensitivity to 1 ppm., acetophenone,[10] benzidine,[11] β-naphthylamine,[12] *p*-methylaminophenol,[13] and *p*-aminoacetophenone.[14] The reaction is familiar as the Koenig reaction. Acetate ion increases the sensitivity of the reaction.[15] Light is a factor in the reaction but primarily if the amine is of high molecular weight. A 2-phase alcohol-water system gives increased sensitivity with benzidine as the color-developing reagent. It has been applied to determination of pyridine and related compounds. Contaminants of waste waters from coking and oil refining are determined in amounts of the order of 0.01 ppm.

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[8] A. A. Shmuk, *Priroda* **1940**, No. 2, 65; Inger Gad and Jens Hald, *Dansk. Tids. Farm.* **15**, 105-17 (1941).

[9] S. B. Tallantyre, *J. Soc. Chem. Ind.* **49**, 466-8T (1930).

[10] A. Tapia Frees, C. Sanchez Monteno, and Cockburn J. Canales, *Actas y trabajos congr. peruno quim.* **2**, I, 337-42 (1943).

[11] R. I. Alekseev, *Zavodskaya Lab.* **8**, 807-9 (1939).

[12] H. von Euler, F. Schlenk, H. Heiwinkel, and B. Hogberg, *Z. physiol. Chem.* **256**, 208-28 (1938).

[13] E. Bandier and J. Hald, *Biochem. J.* **33**, 264-71 (1939).

[14] L. J. Harris and W. D. Raymond. *Ibid.* **33**, 2037-51 (1939); E. G. Wollish, G. P. Kuhnis, and R. T. Price, *Anal. Chem.* **21**, 1412-15 (1949).

[15] W. E. McCormack and H. Smith, *Ind. Eng. Chem., Anal. Ed.* **18**, 508-12 (1946); L. N. Markwood, *J. Assoc. Off. Agr. Chemists* **22**, 427-36 (1939).

after concentration by distillation.[16] The color so developed is reasonably constant at pH 6.2-11.3. Fading is more rapid above pH 9. The optimum sodium acetate concentration is 0.2-0.8 per cent. Light exposure reduces the maximum. α-Naphthylamine and phenols do not interfere at usual concentrations.

Pyridine precipitated as the silicomolybdate is dissolved and reduced to molybdenum blue by sulfite and glycine in alkaline solution.[17] Strong alkali produces a brown color with pyridine in the presence of chloroform.[18] There is no interference by piperidine, quinaldine, pyrroles, and some pyridine homologues. Addition of a solution of a copper salt followed by a solution of thiocyanate precipitates $Cu(C_6H_5N)_2(CNS)_2$. This complex is extractable with difficulty into chloroform for estimation.[19]

Pyridine is developed with barbituric acid for fluorimetric reading.[20] Pyridine is also estimated nephelometrically by phosphotungstic acid and read in sulfuric acid in the ultraviolet at 255 mμ.[21] Homologues may be present.

Sample—*Air.* Pass through a sintered glass distributor into 40 ml. of 1:120 hydrochloric acid. Evaporate to 2 ml. for development as molybdenum blue.

Soil. To an appropriate sample containing 8 mg. of pyridine add 1 gram of benzene, 3 grams of calcium oxide, and 20 grams of sodium chloride in 200 ml. of water. Steam-distil, collecting 150 ml. of distillate in 10 ml. of 1:600 hydrochloric acid. Evaporate the distillate to 2 ml. Develop as molybdenum blue.

Denatured alcohol. Add 1 ml. of 1:120 hydrochloric acid and evaporate to 2 ml. Develop as molybdenum blue.

Ammonium hydroxide. Neutralize a sample containing 25-75 mg. of pyridine. Develop with cyanogen bromide and aniline. Alternatively, nearly neutralize with 1:3 hydrochloric acid. Add 2 drops of 1 per cent

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[16] R. C. Kroner, M. B. Ettinger, and W. Allan Moore, *Anal. Chem.* **24**, 1877-81 (1952).

[17] R. P. Daroga and A. G. Pollard, *J. Soc. Chem. Ind.* **60**, 207-10 (1941).

[18] M. J. Ploquin, *Bull. soc. chim. France* **1947**, 700-1.

[19] G. Spacu, *Bul. soc. Stiinte Cluj* **1**, 284-91 (1922).

[20] E. Asmus and H. Garschagen, *Z. anal. Chem.*, **139**, 81-9 (1953).

[21] Edward Hofmann, *Arch. Hyg. u. Bakt.* **128**, 169-78 (1942); H. D. LeRosen and J. T. Wiley. *Anal. Chem.* **21**, 1175-7 (1949).

nitrophenol solution and titrate with 1:160 hydrochloric acid until colorless. Determine fluorimetrically by barbituric acid.

Petroleum fractions. Measure out a sample containing 25-75 mg. of pyridine and if necessary dilute to 50 ml. with a spectroscopically negative solvent. Extract with 25 ml. and 25 ml. of 10 per cent phosphoric acid for 2 minutes and 1 minute respectively. Dilute the combined acid extracts to 100 ml. with water for reading in the ultraviolet.

Industrial waste. To 500 ml. of sample add a strong solution of sodium hydroxide until the pH is above 10. Distil in glass with chips to prevent bumping and silicones to prevent foaming. Collect 450 ml. of distillate, add 50 ml. of water, and dilute to 500 ml. for determination by cyanogen bromide and benzidine.

Procedure—*By cyanogen bromide and aniline.* Dilute an aqueous sample containing not over 3 mg. of pyridine to 8 ml. with water. Add 2 ml. of 1 per cent aniline in a phosphate buffer for pH 6 (Vol. I, page 178). Add 1 ml. of 10 per cent cyanogen bromide solution. After 5 minutes read at 480 mμ against a reagent blank.

By cyanogen bromide and benzidine. Dilute an aliquot of sample containing 0.001-0.02 mg. of pyridine or equivalent to 100 ml. with water. Add 4 ml. of 15 per cent sodium acetate solution and 2 ml. of 2 per cent benzidine solution slightly acid with hydrochloric acid. Mix and adjust the pH, as falling at 6.8-8. Add 25 ml. of *n*-butanol. As a reagent add bromide dropwise to 100 ml. of 1 per cent potassium cyanide solution under a well-ventilated hood until excess bromine is present. Add 10 per cent potassium cyanide solution dropwise until excess bromine is discharged. Excess bromine must be absent. As a check add 1 ml. of reagent to 2 drops of 1 per cent indigo carmine solution on a spot plate to make sure that the color is not bleached. Add 5 ml. of this reagent to the sample and mix well. Store in the dark for 3.5-4 hours at room temperature. Remove 5 ml. of the butanol layer and read at 520 mμ against a reagent blank.

As molybdenum blue. As precipitating agent dissolve 14.4 grams of molybdic anhydride in 100 ml. of 4 per cent sodium hydroxide solution. Add a solution of sodium silicate containing 0.7 gram of silica and slowly add 1:3 hydrochloric acid with stirring until the solution turns green. Dilute to about 900 ml. and heat on a steam bath for 3 hours. After standing at room temperature for 24 hours, filter off excess silica

and add 2.5 grams of cupric chloride dissolved in water. Dilute to 1 liter and keep in a dark bottle.

Mix 1 ml. of sample solution, 1 ml. of 1:120 hydrochloric acid, and 1 ml. of reagent. Warm and shake to coagulate the precipitate. Filter on paper in a Gooch crucible after an hour and wash the precipitate twice with 0.5-ml. portions of a 10 per cent solution of sodium chloride in 1:120 hydrochloric acid. Dissolve the precipitate from the filter and the original beaker with 2 ml. of a solution containing 1 gram of glycine in 15 ml. of 20 per cent sodium sulfite, into which 5 ml. of concentrated ammonium hydroxide has been stirred. Wash with more reducing solution, dilute to 50 ml., and store at 45° for 25 minutes. Read at 610 mμ against a reagent blank.

By alkali. Mix 5 ml. of water saturated with ether, 2 pellets of potassium hydroxide, and 2.5 ml. of chloroform. Add a sample containing 10-100 mg. of pyridine and place in a 75° bath until the chloroform is gone. Read the yellow to brown color against water.

In the ultraviolet. Read at 255 mμ against a reagent blank.

Fluorimetrically by barbituric acid. Add successively 2 ml. of 1:160 hydrochloric acid, 1 ml. of 1 per cent potassium cyanide solution, and 5 ml. of 1 per cent solution of ammonium chloride. Mix, add 5 ml. of sample, and mix. After 5 minutes add 10 ml. of 1 per cent solution of barbituric acid with mixing. Read the fluorescence after 30 minutes against water.

Piperidine, Hexahydropyridine

The effect of piperidine in permanently decolorizing triphenylmethane dyes such as malachite green is used for its quantitative estimation.[22]

Procedure—To 1 ml. of sample solution add 0.5 ml. of 0.1 per cent solution of malachite green in freshly distilled pyridine. Follow with 2.5 ml. of methanol. Heat at 60-70° for 1-2 minutes and cool. Read at 610 mμ against a reagent blank.

4-Picoline

A reaction with 1-chloro-2,4-dinitrobenzene is described under isonicotinic acid hydrazide (page (000). Follow the procedure given there but read at 560 mμ. Results are reproducible to ±2 per cent.

[22] L. M. Kul'berg and I. S. Mustafin, *Zhur. Anal. Khim.*, 7, 84-8 (1952).

2-Aminopyridine

Although the usual methods for estimation of 2-aminopyridine are titrametric or by Kjeldahl determination, the spectrophotometric absorption shows a maximum at 287 mμ.[23]

Pyridine-3-Carboxylic Acid, Nicotinic Acid, Niacin

The determination of niacin is included in nitrogen-containing cycles because the most-used procedure depends on the pyridine grouping. That grouping reacts with cyanogen bromide and may thereafter be reacted with an amine.[24] Closely analogous reactions are given by niacinamide. Pyridoxine gives a colored compound which interferes if heat is used, but at room temperature this is not produced.[25] The product of reaction between a pyridine derivative and cyanogen bromide in acid solution causes absorption maxima for pyridine of 378.3 mμ, niacin 352.4 mμ, niacinamide 392.5 mμ, coramine 393.2 mμ, and nicotine 378 mμ.[26] The reaction takes place quickly at 75-80° but decreases rapidly from the maximum. Greater intensity is developed at 25° in 30 minutes. The corresponding reaction in alkaline solution gives a visible color. The product produced in acid solution contains no bromine.[27]

Considering both visible and ultraviolet absorption, the maximum intensity is developed at pH 3-4 with a lesser intensity at pH 8. A citrate or acetate buffer intensifies the absorption at the higher pH. The reactions at the two pH levels are fundamentally different.

By further treatment with an amine, the ultraviolet absorption developed at the lower pH is brought into the visible range. This reagent does not determine reduced nicotinic acid.[28] Nucleotides must be hydrolyzed.

Various amines applied to develop the color include aniline,[29]

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[23] W. O. Winkler, *J. Assoc. Offic. Agr. Chemists* **31**, 760-9 (1948).

[24] W. König, *J. prakt. Chem.* **69**, 105 (1904).

[25] Arthur E. Teeri, *Chemist-Analyst* **41**, 62-4 (1952).

[26] Michael Vacher and Odette Tounichon, *Bull. soc. chim. biol.* **31**, 1430-8 (1949).

[27] E. Samuels Brusse, *Pharm. Weekblad* **85**, 569-88 (1950); Cf. Hideo Higashi and Massao Hirai, *J. Agr. Chem. Soc. Japan* **20**, 15-22 (1944).

[28] S. A. Singal, V. P. Sydenstricker, and J. M. Littlejohn, *J. Biol. Chem.* **176**, 1069-73 (1948).

[29] M. Swaminathan, *Nature* **141**, 830 (1938); *Indian J. Med. Research* **26**, 427-34 (1938); *Ibid.* **30**, 397-401 (1942); *Ann. Biochem. Exptl. Med.* **6**. 69-74 (1946); H. E. Shaw and C. A. MacDonald, *Quart. J. Pharm. and Pharmacol.* **11**, 380-90

p-aminoacetophenone,[30] *p*-methylaminophenol sulfate [31] or Metol, sulfanilamide,[32] *m*-phenylenediamine hydrochloride,[33] sulfanilic acid,[34] or 2-naphthylamine sulfonic acid (Tobias acid).

Typical maxima for absorption are Metol 405μ, m ammonia 410 mμ, aniline 430 mμ, sulfanilic acid 450 mμ, and 2-naphthylamine-1-sulfonic acid 500 mμ. The higher the wave length of maximum absorption the less the interference by background color. Sulfanilamide develops a much more intense color than aminoacetophenone. The degree of interfering color in the sample suggests the desirability of extraction of the developed color with ethyl acetate, amyl acetate, etc. This avoids removal of interfering colors with activated carbons which, while sorbing no niacin from strongly acid alcoholic solution, does under the usual conditions. Also residual color is darkened by the reagent to a variable degree, introducing an error. When developed by aniline, piperidine, pyrrole, quinoline, 2-methylquinoline, pyridine, and furfur-

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(1938); Hans von Euler, Fritz Schlenk, Heinz Heiwinkel, and Bertil Högberg, *Z. physiol. Chem.* **256**, 208-28 (1938); Hans Kringstad and Thomas Naess, *Naturwissenshaften* **26**, 709 (1938); *Z. physiol. Chem.* **260**, 108-18 (1939); P. B. Pearson, *J. Biol. Chem.* **129**, 491-4 (1939); E. Askelof and C. Holmberg, *Svensk. Farm. Tids* **43**, 301-7, 321-6 (1939); K. Ritsert, *Klin. Wochschr.* **18**. 934-6 (1939); I. G. Porje, *Nord. Med.* **2**, 1108-10 (1939); Daniel Melnick and Henry Field, Jr., *J. Biol. Chem.* **134**, 1-16 (1940); B. D. Kochhar, *Indian J. Med. Research* **28**, 385-96 (1940); Harry H. Waisman and C. A. Elvehjem, *Ind. Eng. Chem., Anal. Ed.* **13**, 221-5 (1941); Daniel Melnick, Bernard L. Oser and Louis Siegel, *Ibid.* **13**, 879-82 (1941); Daniel Melnick, *Cereal Chem.* **19**, 553-67 (1942); J. Abdon and K. Täufel, *Z. Untersuch Lebensm.* **84**, 385-92 (1942); Bashir Ahmad and Amar Nath Sharma, *Ann. Biochem. Exptl. Med.* **6**, 35-40 (1946); S. Banerjee, N. C. Ghosh, and G. Bhattacharya, *J. Biol. Chem.* **172**, 495-9 (1948).

[30] L. H. Harris and W. D. Raymond, *Biochem. J.* **33**, 2037-51 (1939); E. Kodicék, *Ibid.* **34**, 712-35 (1940); Aaron Arnold, C. B. Schreffler and S. T. Lipsius, *Ind. Eng. Chem., Anal. Ed.* **13**, 62-3 (1941); C. I. Noll and O. G. Jensen, *J. Biol. Chem.* **140**, 755-62 (1941); Y. L. Wang and E. Kodicék, *Biochem. J.* **37**, 530-8 (1943); J. C. Roggen, *Mededeel. Lab. Physiol. Chem. Univ. Amsterdam En Nederland. Inst. Volksvoed.* **10**. No. 18, 44 pp. (1945-7).

[31] E. Bandier, *Biochem. J.* **33**, 1130-40 (1939); E. Bandier and Jens Hald, *Ibid.* **33**, 2647-71 (1939); L. A. Rosenblum and N. Jolliffe, *J. Biol. Chem.* **134**, 137-41 (1940); W. J. Dann and Philip Handler, *Ibid.* **140**, 201-13 (1941); Elmer Stotz, *J. Lab. Clin. Med.* **26**, 1042-6 (1941).

[32] Yves Raoul and O. Crépy, *Bull. soc. chim. biol.* **23**, 362-75 (1941); Yves Raoul, *Ann. pharm. franc.* **1**, 17-20 (1943).

[33] Arthur E. Teeri and Stanley R. Shimer, *J. Biol. Chem.* **153**, 307-11 (1944).

[34] James P. Sweeney and Wallace L. Hall, *Anal. Chem.* **23**. 983-6 (1951); J. P. Sweeney, *J. Assoc. Official Agr. Chemists* **34**, 380-7 (1951).

aldehyde interfere. When developed by Metol in the presence of monopotassium phosphate there is no interference from moderate amounts of pyridine, picolinic acid, α-picoline, trigonelline, or methyl pyridinium chloride. While niacinamide gives the reaction, the intensity of coloration is reduced by approximately one-half with aniline but increased with Metol. Therefore niacinamide is frequently hydroyzed to niacin for determination by the same method.

The brown pigment of hydrolyzed tissue interferes with the method.[35] The best method of deproteinizing is with lead acetate and sodium sulfide.

Cyanogen bromide has commonly been made by adding cold 10 per cent sodium cyanide solution to a cold saturated bromine solution until just colorless. The eye does not detect the end point with sufficient accuracy, excess bromine sometimes giving an off-color by oxidation of the amine, excess cyanide affecting the buffering for pH adjustment.[36]

Another method of estimation consists of fusion of the niacin or niacinamide with 2,4-dinitrochlorobenzene to form a quaternary pyridinium salt.[37] On decomposition with alkali, the yellow-red derivative of glutoconaldehyde is read promptly before fading occurs. Extraction of the excess of reagent with ether removes some of the reaction product.[38] Creatine also gives the color[39] and that color is altered on dilution. Niacin is also estimated by molybdenum blue developed from phosphomolybdic acid[40] and by the yellow from monochloramine followed by potassium cyanide.[41]

For differentiation of niacin and niacinamide, the differential rate of color development with sulfanilic acid is used, or they are separated by paper chromatography.[42]

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35 Kazuo Kawashima, *J. Japan. Biochem. Soc.* **19**, 144-59 (1947).

36 Harold C. Goldthorpe and Doris Tippitt, *Anal. Chem.* **23**, 484-7 (1951).

37 S. P. Vilter, T. D. Spies and A. P. Mathew, *J. Biol. Chem.* **125**, 85-98 (1938); *J. Am. Chem. Soc.* **60**, 731-2 (1938); Paul Karrer and H. Keller, *Helv. Chim. Acta* **21**, 463-9, 1170-1 (1938); *Ibid.* **22**, 1292-3 (1939); Mario Covello, *Boll. soc. ital. biol. sper.* **13**. 1021-3 (1938).

38 Walter R. Ashford and R. H. Clark, *Trans. Roy. Soc. Can.* **III, 33**, 29-32 (1939).

39 Leo Schindel, *J. Lab. Clin. Med.* **25**, 515-16 (1940).

40 R. P. Daroga, *J. Soc. Chem. Ind.* **60**, 263-6 (1941).

41 F. Yu Rachinskiĭ, Ya. M. Slobodin, and I. N. Shakhov, *J. Applied Chem. (USSR)* **19**, 176-9 (1946).

42 E. G. Wollish, Morton Schmall, and E. G. E. Schafer, *Anal. Chem.* **23**, 768-71 (1951).

Samples—*Animal tissue.* Digest a mixture of 1-5 grams of minced tissue, 0.5 gram of pepsin, and 50 ml. of 1:120 hydrochloric acid at 37° for 24 hours. Add 5 ml. of 4 per cent sodium hydroxide solution to approximate pH 5.2 and dilute to 100 ml. Add 6 ml. of 4 per cent sodium hydroxide solution to 10 ml. of the acid-pepsin digest, which should make it 0.38 *N* with a pH about 12. Heat in boiling water for 1 hour to hydrolyze any niacinamide diphosphopyridine nucleotide and triphosphopyridine nucleotide to nicotinic acid.

Partially neutralize by addition of 5 ml. of 1:11 hydrochloric acid. Add 2 ml. of 10 per cent zinc sulfate solution to the partially neutralized alkaline digestate and dilute to 25 ml. Mix well, let stand for 10 minutes, centrifuge, and decant the upper layer. For successful use this must have a pH of 7-7.2 Use as the sample solution for development with cyanogen bromide and Metol.

Alternatively hydrolyze 8-16 grams with 4 ml. of 40 per cent sodium hydroxide solution by stirring at 100° for 30 minutes. This liberates niacin from codehydrases such as diphosphopyridine-or triphosphopyridine-nucleotide. Centrifuge and mix 10 ml. of the upper layer with 2 ml. of 40 per cent lead acetate solution. Add 1 ml. of 40 per cent sodium sulfide solution, mix, and centrifuge. Adjust to pH 6 for development with cyanogen bromide and aniline.

Plant tissue. Heat 8-16 grams for 1 hour with 4 ml. of concentrated hydrochloric acid. Cool and neutralize with 40 per cent sodium hydroxide solution and add 4 ml. excess. Heat at 100° for 30 minutes. Complete as for the second method for animal tissue from "This liberates niacin . . . ."

Feces.[43] Disperse a sample expected to contain 0.03-0.04 mg. of niacin. Complete as for the first method for animal tissue, starting at "Add 5 ml. of 4 per cent sodium hydroxide solution . . . ."

Urine. From a 24-hour specimen preserved with thymol in the refrigerator use aliquots for development of the color with cyanogen bromide and Metol. When essential, sorb excessive color on zinc hydroxide [44] or a rare-earth hydroxide.[45]

Alternatively,[46] boil 40 ml. of urine with 5 ml. of 40 per cent sodium

[43] Vittorio Zambotti, Atti. soc. med.-Chir. Padova, **19**, 827-9 (1942).

[44] Theodore E. Friedemann and Clifford J. Barborka, *J. Biol. Chem.* **128**, 785-6 (1941); K. I. Kokovikhina, *Biokhimiya* **11**, 63-70 (1946).

[45] Walter Ciusa and Walter Pasquali, *Ann. chim. applicata* **36**, 167-78 (1946).

[46] J. Kuhnau and K. Gaede, *Klin. Wochschr.* **23**, 332-4 (1944).

hydroxide solution for 30 minutes to hydrolyze all esters. Acidify with concentrated hydrochloric acid and add 10 ml. of 30 per cent lanthanum nitrate solution. Dilute to 60 ml., filter, and to 45 ml. of filtrate add 5 ml. of 10 per cent sodium hydroxide solution and 50 ml. of methanol. Filter the precipitate of lanthanum hydroxide and evaporate the filtrate to about 50 ml. Develop with cyanogen bromide and an amine.

Blood.[47] The concentration of niacin in blood cells is more than ten-fold that in plasma, therefore calling for different methods of treatment.

Blood cells. Mix 2 ml. of cells, obtained from oxalated blood, with 12 ml. of water. After 10 minutes, add 3 ml. of 1:50 sulfuric acid, mix, and add 5 ml. of 10 per cent sodium tungstate dihydrate solution. Shake vigorously, stopper loosely, and place in boiling water for 20 minutes. Mix frequently during the first 5 minutes to prevent the precipitate from collecting at the top of the fluid. Cool to room temperature, centrifuge, and filter the supernatant liquid.[48] A technic closely analagous to that which follows has been applied to foodstuffs.

Heat 10 ml. of filtrate mixed with 2 ml. of concentrated hydrochloric acid for 1.5 hours in boiling water, cool, and make the solution alkaline to phenolphthalein. Adjust the pH to approximately 5 to indicator paper by dropwise additions of 1:2 hydrochloric acid. Use an aliquot for development with cyanogen bromide and Metol.

Plasma. Prepare a protein-free filtrate from 10 ml. of plasma and 2 ml. of water by the method described for analysis of cells through ". . . filter the supernatant liquid."

Hydrolyze by heating 10 ml. of filtrate with 2 ml. of concentrated hydrochloric acid for 1.5 hours. Dilute the hydroyzate to 12 ml., mix, and centrifuge. Add 1 ml. of approximately 60 per cent potassium hydroxide solution to 11 ml. of the supernatant fluid. Adjust the pH of the solution to 0.5-1, and add 0.5 gram of Lloyd's reagent. Mix carefully to liberate gas and then shake for a short time. Wash the Lloyd's reagent down from the sides with a few drops of 1:180 sulfuric acid,

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[47] Guimarães Villela, *O Hospital* **17**, 431-42 (1940); Henry Field Jr., Daniel Melnick, William D. Robinson and Charles F. Wilkinson, *J. Clin. Investigations* **20**, 379-86 (1941); Harris Isbell, Jerald G. Wooley, R. E. Butler and W. H. Sebrell, *J. Biol. Chem.* **139**, 499-510 (1941); J. Raymond Klein, William A. Perlzweig and Philip Handler, *Ibid.* **45**, 27-34 (1942); Cf. J. C. Roggen, *Nederland, Tijdschr. Geneeskinde* **85**, 4603-8 (1941); *Rec. trav. chim.* **62**, 137-47 (1943).

[48] Estelle Hausmann, Lawrence Rosner, and Howard J. Cannon, *Cereal Chem.* **20**, 82-6 (1943).

centrifuge, and discard the supernatant liquid. Add 4 ml. of about 2.8 per cent potassium hydroxide solution to the Lloyd's reagent, mix, add a drop of phenolphthalein, and dilute with water to 12 ml. Centrifuge, collect the supernatant fluid, and add to 0.3 gram of powdered lead nitrate. Mix thoroughly. Adjust the pH so that the solution is not quite alkaline to phenolphthalein. Centrifuge and collect the supernatant liquid. Add solid tripotassium phosphate until the solution is just alkaline to phenolphthalein. Centrifuge and pipet off 5 ml. of solution. Adjust the pH to about 5 to indicator paper by dropwise addition of 1:2 hydrochloric acid solution. Use an aliquot for development by cyanogen bromide and Metol.

Biological materials in general.[49] Mix a sample containing 0.01-0.4 mg. of niacin with 5 ml. of concentrated hydrochloric acid and dilute with water to 15 ml. Immerse in boiling water for 30-40 minutes with occasional stirring. Cool to room temperature and adjust the volume to 15 ml. Add 10 ml. of absolute ethanol, shake with 0.2 gram of activated carbon and filter. Neutralize 8.33 ml. of the filtrate to pH of about 5 with 10 per cent sodium hydroxide solution in the cold. Dilute to 10 ml. for development with cyanogen bromide and aniline.

Beef, wheat germ, yeast, rice bran. Suspend a sample containing about 0.4 mg. of niacin in 75 ml. of water in a centrifuge bottle and autoclave for 15 minutes at 15 pounds per square inch. Cool, centrifuge, and decant the supernatant liquid. Dilute the residue with sufficient water to make the total extract about 80 ml., centrifuge, and decant. Make the combined extracts alkaline with 5 ml. of 20 per cent sodium hydroxide solution and heat for 30 minutes on a steam bath to liberate niacin from any amide present. Cool and add 2 ml. of 4 per cent sodium bicarbonate solution. Add 1.5 ml. of concentrated hydrochloric acid and adjust the pH to 6-6.5 with 1:3 hydrochloric acid, using thymol blue as an external indicator. Dilute to 100 ml. for the development of aliquots with cyanogen bromide and *p*-aminoacetophenone.

Plant Materials.[50] *Seed portion.* Heat a 0.5-1.5-gram sample with water on a water bath for 45 minutes with occasional stirring. Cool to room temperature, dilute to 15 ml., and centrifuge. Hydrolyze 5 ml. of this aqueous extract with 1 ml. of 20 per cent sodium hydroxide solution in boiling water for 5-10 minutes. Cool and add about 40 ml.

[49] Daniel Melnick and Henry Field, Jr., *J. Biol. Chem.* **134**, 1-16 (1940).

[50] E. B. Hale, G. K. Davis and H. R. Baldwin, *Ibid.* **146**, 553-63 (1942); Cf K. V. Giri and B. Naganna, *Indian J. Med. Research* **29**, 125-32 (1941).

of ethanol to precipitate interfering substances. Centrifuge and decant. Add 1 ml. of 5 per cent sodium bicarbonate solution to the extract and bring to pH 6 by adding concentrated hydrochloric acid dropwise. Dilute to 50 ml. with ethanol for development of aliquots with cyanogen bromide and *p*-aminoacetophenone.

Forage portion. Prepare the extract to ". . . dilute to 15 ml., and centrifuge." Hydrolyze 5 ml. of this aqueous extract with 1 ml. of concentrated hydrochloric acid for 30 minutes in boiling water. Cool and add about 40 ml. of ethanol to precipitate interfering substances. Adjust to pH 6 by careful addition of 30 per cent sodium hydroxide solution. Dilute to 50 ml. with ethanol and develop by cyanogen bromide and *p*-aminoacetophenone.

Milk Powders. Dissolve a 25-gram sample in water and dilute to 200 ml. Mix a 50-ml. aliquot with 2.5 ml. of concentrated sulfuric acid and heat the sample containing 8 per cent of sulfuric acid on the steam bath for 1 hour. Cool to room temperature, centrifuge, and decant the supernatant extract. Wash the residue into the original beaker with 40 ml. of 1:19 sulfuric acid and heat for 1 hour on the steam bath. Cool, centrifuge as before, and decant the supernatant liquid into the previous extract. Treat the residue in the same manner as before, using 30 ml. of 1:19 sulfuric acid. Adjust the pH of the combined extracts to 6-6.2 with 70 per cent sodium hydroxide solution with a glass electrode and dilute to 150 ml. Use a 10-ml. aliquot for development by cyanogen bromide and *p*-aminoacetophenone. Do not add *p*-aminoacetophenone to the blank. Calculate the niacin from a standard run at the same time.

Flour and Bread.[51] Autoclave a 2-gram sample of flour or air-dried bread with 100 ml. of water for 30 minutes at 15-pounds pressure. Centrifuge and wash the precipitate twice with 50 ml. of boiling water. Add 10 drops of caprylic alcohol to prevent foaming and concentrate the extract and washings *in vacuo* or in an over at 115° to 5 ml. or less. Add 5 ml. of concentrated hydrochloric acid and immerse in boiling water. Dilute to 15 ml. with water previously used to rinse the flask. Immerse in boiling water for 30-40 minutes and stir occasionally. Cool to room temperature and dilute to 15 ml. Add 10 ml. of absolute ethanol and shake with 0.3 gram of activated carbon. Filter at room

[51] Daniel Melnick, Bernard L. Oser and Louis Siegel, *Ind. Eng. Chem., Anal. Ed.* **13**, 879-83 (1941); Cf. Hans Kringstad and Thomas Naess, *Z. physiol. Chem.* **260**, 108-18 (1939).

temperature. Neutralize a 12.5 ml. aliquot with 10 per cent sodium hydroxide solution in the cold to pH 7. Dilute to 15 ml. and develop by cyanogen bromide and aniline.

Alternatively,[52] stir 20 grams of flour gradually into 200 ml. of boiling water and autoclave at 15 pounds per square inch for a half hour. Centrifuge and decant. Add 200 ml. of water to the residue, disperse, and autoclave. Separate and repeat once more. Evaporate the combined extracts nearly to dryness in boiling water. Add 20 ml. of concentrated hydrochloric acid and heat for an hour in boiling water. Neutralize the concentrate so obtained with 16 per cent sodium hydroxide solution and adjust the pH to 5. Dilute to 20 ml. and centrifuge. Mix 1 ml. of the clear upper layer with 9 ml. of acetone, stir for 3 minutes, and centrifuge. Mix 4.5 ml. of the clear layer with 3 ml. of water and evaporate *in vacuo* without heating. All acetone must be removed. Use this as sample for development by cyanogen bromide and Metol.

Enriched food products and feeds. Autoclave 25 grams of sample with 200 ml. of 1:144 sulfuric acid at 15 pounds' pressure for 15 minutes. Cool and add 40 per cent sodium hydroxide solution to adjust to pH 4.5 with bromocresol green as an outside indicator. This precipitates the proteins at their isoelectric point. Filter and dilute to 250 ml. To 40 ml. of this solution add 17 grams of ammonium sulfate, dissolve, and dilute to 50 ml. Develop 2 ml. by cyanogen bromide and sulfanilic acid.

For development by cyanogen bromide and 2-naphthylamine-1-sulfonic acid proceed through precipitation of protein, filtration, and dilution to 250 ml. Heat a 30-ml. aliquot to boiling and add 1:2 ammonium hydroxide dropwise to a strong odor of ammonia. Heat for 2 minutes, filter, and adjust to a pH of 4.5. Add to 22 grams of ammonium sulfate and dilute to 50 ml. Filter and develop an aliquot.

Tablets. Grind at least 5 tablets and dissolve in a little water by heating. Dilute to a known volume to contain 0.05-0.2 mg. per ml. and take a 10 ml. aliquot as sample. Develop by cyanogen bromide and sulfanilic acid or 2-naphthylamine-1-sulfonic acid.

Capsules. Disperse 5 or more in water and treat as for tablets, starting at "Dilute to a known volume . . . ."

Separation from niacin. The apparatus is shown in Figure 11. A borosilicate tube about 55 cm. long is closed at one end and the other end joined to a 6-inch borosilicate glass flange with a ground surface.

[52] Joses M. Montanes del Olivo, *Anales fis. y quim.* (Madrid) 42, 255-64 (1946).

The solvent container is a 100-ml. beaker with the rim removed and one side flattened. A microscope slide is attached to the flattened side of the beaker by a wire. Two such beakers, each supporting a paper strip, are used with one tube on opposite sides. Place about 50 ml. of ethyl acetate saturated with water in the bottom of the tube and the same solvent in each beaker.

Develop the chromatograph on 3.12×55 cm. strips of Whatman No. 1 paper by applying exactly 0.1 or 0.2 ml. of sample about 3 inches from one end as indicated in the figure. Use the lower part of a 6-inch

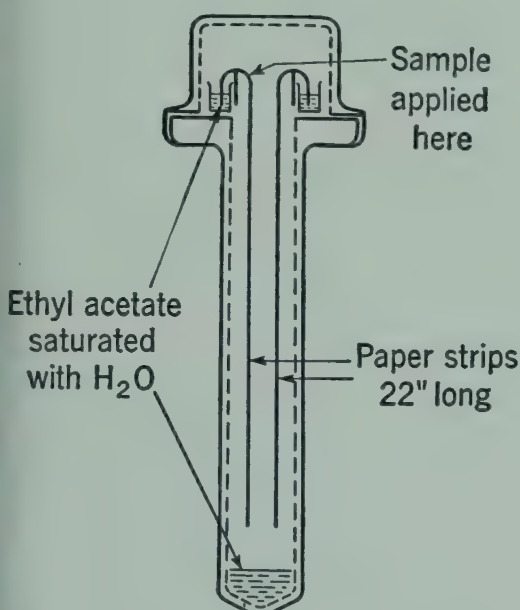

FIG. 11. Apparatus for chromatography

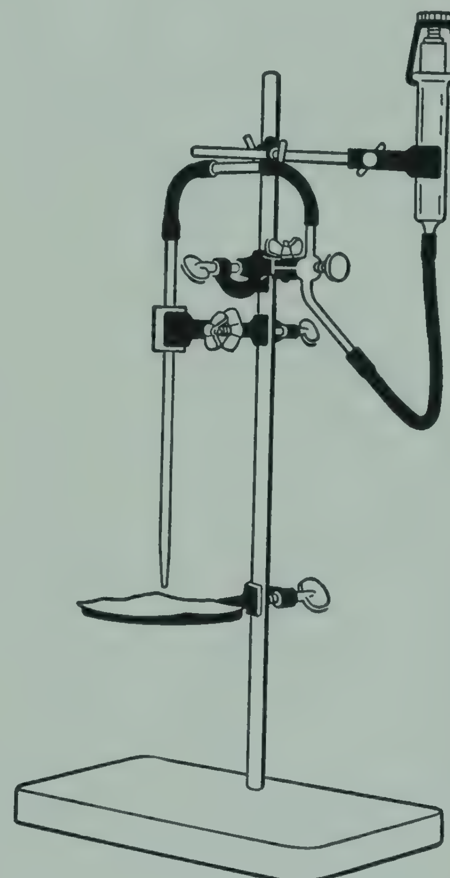

FIG. 12. Apparatus for delivery of sample

desiccator as a cover to insure vapor saturation. To get a good chromatogram, it is essential that the sample be applied in a very narrow band. The requisite apparatus as shown in Figure 12 consists of a 0.2-ml. micropipet connected ton an all-metal needle valve and in turn to a 30-ml. glass hypodermic syringe. The piston is attached to the cylinder by a coil spring to provide constant pressure.

To fill the pipet, open the valve and withdraw the plunger of the

syringe half way. Place the tip of the pipet in the solution and draw up to above the zero mark. Close the valve and adjust to the zero mark by opening it. Place a drop of solution on the paper and close the valve. When this has evaporated, add another drop and repeat until the solution has been added and evaporated.

Then, with the strip in place, in 3 hours at 25° the solvent front moves down 16 inches. Remove the paper, air-dry, and cut into 1-inch sections. Places each in 1 ml. of phosphate buffer for pH 6 (See Vol. I, page 174) and add 4 ml. of water. Develop with cyanogen bromide and *p*-aminoacetophenone. All of the niacin is in the first inch of paper, the niacinamide at around 5-7 inches. By this technic one part of niacin can be separated from 100 parts of niacinamide.

Procedure—*By cyanogen bromide and Metol.* Set up tubes calibrated at 25 ml. according to the following table. All values are in ml.

Tube	*Aliquot*	*Buffer*	*Cyanogen Bromide Reagent*	*Amine Reagent*	*Water*
1	0	3	4	14	4
2	0	3	4	14	4
3	3	0	0	0	22
4	3	0	0	0	22
5	3	3	4	14	1
6	3	3	4	14	1
7	3	3	4	0	0

Thus tubes 1 and 2 are reagent blanks and 3 and 4 are sample blanks. Tube 7 is to control the pH before adding the reagents to tubes 5 and 6.

Determine the pH in tube 7 by the glass electrode. If higher than 5.1, adjust with a drop or two of 10 per cent citric acid solution, if below add a drop or two of 10 per cent dibasic sodium phosphate. Similarly treat the other tubes without measurement of pH.

As buffer, use a solution containing 1.52 per cent of disodium hydrogen phosphate and 0.975 per cent of citric acid monohydrate. Check the pH and adjust if necessary to 5.25. Add as indicated. After adjustment of pH add 4 per cent cyanogen bromide reagent, prepared from the crystalline salt, to the tubes and let them stand in the dark at 25° for 30 minutes.

If the Metol is pink, wash the solid with ethanol. Prepare a 5 per cent solution in 1:360 sulfuric acid. Cool the tubes and amine reagent

to 10-15° and add the volume of the latter indicated in the table. Dilute to volume, store in the dark at 10-15° for 80 minutes, and read at 410 mμ against water. Subtract the averages of tubes 1 and 2, and of tubes 3 and 4, from the average of tubes 5 and 6. Thus the reading is corrected for both a reagent blank and a sample blank. Also correct for any niacin in the pepsin used in preparation of protein samples.

By cyanogen bromide and p-aminoacetophenone. Aqueous extracts. Use a 5-ml. aliquot of the prepared extract. Heat in water at 80° for 10 minutes. Add 3 ml. of 4 per cent aqueous cyanogen bromide prepared from the colorless salt and maintain at 80° for exactly 5 minutes. Cool rapidly to room temperature and after 2 minutes add 0.5 ml. of 5 per cent *p*-aminoacetophenone solution in ethanol. Mix and add 0.6 ml. of 1:3 hydrochloric acid. Allow to stand in the dark for 15 minutes.

Shake the reaction mixture mechanically with 13 ml. of ethyl acetate for 7 minutes. Allow the layers to separate, and centrifuge to clear the emulsion if necessary. Discard the lower layer and clarify the ethyl acetate by adding 2 grams of anhydrous sodium sulfate. Read the clarified ethyl acetate solution at 420 mμ against a blank from which the cyanogen bromide was omitted.

Ethanol solutions. Heat 10 ml. of extract at 70-80° for ten minutes. Add 3 ml. of 4 per cent cyanogen bromide solution. After 5 more minutes, cool to room temperature with tap water. Add 2 ml. of 5 per cent *p*-aminoacetophenone in 3:7 hydrochloric acid. Mix well, store in the dark, and read at 420 mμ after 5 minutes. Correct by application of a reagent blank in which 1:10 hydrochloric acid replaced the cyanogen bromide.

By cyanogen bromide and aniline. Prepare an alcoholic buffer containing 1 ml. of 15 per cent sodium hydroxide solution, 33.3 ml. of absolute ethanol, and 196 ml. of water. Mix 3 ml. of sample with 7 ml. of this buffer as a blank. Mix 3 ml. of sample, 6 ml. of 4 per cent cyanogen bromide solution, and 1 ml. of 4 per cent aniline in absolute ethanol. Read at 420 mμ at the end of 5 minutes. Subtract the blank which measures the color not removed in preparation of the sample.

Niacin and niacinamide by cyanogen bromide and sulfanilic acid. Treat an aliquot of sample solution containing about 1 mg. of nicotinic acid and nicotinamide with 10 ml. of concentrated hydrochloric acid and evaporate on a hot plate to about 2 ml. Add 50-75 ml. of water and then add 10 per cent sodium hydroxide until alkaline to bromothyol blue on a spot plate. Dilute to about 0.01 mg. per ml. Similarly dilute

another aliquot of the unhydrolyzed solution to the same volume. Prepare 20 per cent sulfanilic acid solution adjusted to pH 4.5 with concentrated ammonium hydroxide.

Prepare a group of tubes according to the following table.

	1	2	3	4	5	6	7
Niacin standard, 0.01 mg. per ml., ml.	1.0	1.0	—	—	—	—	—
Niacinamide standard, 0.01 mg. per ml., ml.	—	—	1.0	—	—	—	—
Unhydrolyzed sample, ml.	—	—	—	1.0	1.0	—	—
Hydrolyzed sample, ml.	—	—	—	—	—	1.0	1.0
Water, ml.	6.5	1.5	1.5	6.5	1.5	6.5	1.5
1:5 ammonium hydroxide, ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5
10 per cent aqueous cyanogen bromide, ml.	—	5.0	5.0	—	5.0	—	5.0
Sulfanilic acid reagent, ml.	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Concentrated hydrochloric acid, drops	1.0	—	—	1.0	—	1.0	—
Water, drops	—	1.0	1.0	—	1.0	—	1.0

Add the sample or standard solution, water and ammonium hydroxide. Then add the sulfanilic acid and hydrochloric acid to tube 1 and use at 430 mμ to set the instrument. Add the cyanogen bromide solution under a hood to a sample or standard with swirling and at once add the sulfanilic acid solution. Read in 1.5-2 minutes.

Correct 5 with the unhydrolyzed blank 4, 7 with the hydrolyzed blank 6.

For calculation the values in parentheses are the readings of the numbered tubes.

$$\frac{(2) - (3)}{0.01} = \text{(x) difference in absorbancy of 0.01 mg. of niacin and 0.01 mg. of niacinamide.}$$

$$\frac{(2) - (5)}{(x)} = \text{mg. niacinamide in unhydrolyzed sample.}$$

$$\frac{(5) - [(5) - (7)]}{2(x)} = \text{mg. niacin in unhydrolyzed sample.}$$

By cyanogen bromide and 2-naphthylamine-1-sulfonic acid. As reagent disperse 10 grams of Tobias acid in about 75 ml. of water at not over 70°. Add 40 per cent sodium hydroxide solution dropwise until solution is complete. Adjust the pH to 4.5 to bromocresol green as an outside indicator by addition of 1:1 hydrochloric acid. Decolorize by warming with about 1 gram of activated carbon for about 5 minutes, filter, and dilute to 100 ml. Stored in a dark bottle this keeps only a few days.

Treat 1 ml. of sample containing about 0.012-0.015 mg. of niacin with 1 ml. of buffer containing 7 per cent of monopotassium phosphate and 4 per cent of disodium phosphate dodecahydrate. After exactly 30 seconds, add 5 ml. of 10 per cent aqueous cyanogen bromide prepared from crystals with vigorous agitation. Prepare and use this under a hood. Stopper and place in a dark cabinet with appropriate blanks. Read at 15-30 minutes against a reagent blank. This technic requires greater care than the sulfanilic acid procedure, but the color is more stable and more easily read.

2-Pyridoxic Acid, Isonicotinic Acid, 2-Methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine

Pyrodoxic acid is an oxidation product of pyridoxine determined by fluorescence either directly or as the lactone.[53] Coumarin or vanilla metabolize to give interfering substances. By reading as the lactone and then breaking the ring with alkali and reading again, a more representative blank is obtained.[54] The fluorescence of the lactone is about 25 times as great as of the free acid.

Sample—*Urine.* Dilute a 1-ml. sample to 10 ml. with 1:10 hydrochloric acid and heat in boiling water for 15 minutes. Cool and dilute to a known volume. Buffer around pH 9 by adding solid sodium borate and read with Coleman filters B1 and PC1 as used for determination of thiochrome. Use a standard containing 0.04 gamma of the lactone. Adjust the pH to about 12 with 30 per cent sodium hydroxide solution and heat in boiling water to break the lactone ring. Cool and read as the blank to be subtracted.

Isonicotinic Acid Hydrazide

The colored reaction product of isonicotinic acid hydrazide and β-naphthoquinone-4-sulfonate in the presence of sodium hydroxide is read at 480 mμ.[55] The reaction is with the hydrazide substituent. Semicarbazide gives the same color at much lower intensity. Hydroxylamine gives a maximum at 440 mμ with this reagent. Other chromophores with absorption maxima around 440 mμ are given by hydrazine,

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53 Jesse W. Huff and William A. Perlzweig, *J. Biol. Chem.* **155**, 345-55 (1944).

54 Poul Møller, *Acta Chem. Scand.* **5**, 1418-20 (1951).

55 Sidney Stern and Thomas H. McGavack, *Anal. Chem.* **25**, 813-14 (1953).

phenylhydrazine, and aniline. Absorption around 480 mμ is given by urea. There is no color development with acetamide, acetanilide, picolinic acid, ethyl nicotinate, and pyridine.

The purple color of isonicotinic acid hydrazide with 1-chloro-2, 4-dinitrobenzene is read at 530 mμ.[56] A similar color with a maximum at 560 mμ is given by 4-picoline. There is no corresponding color from 2-picoline, 3-picoline, 2,6-lutedine, pyridine, isonicotinic acid, or ethyl isonicotinate. Results are reproducible to ±3 per cent.

If a sample is brought to pH 10 with sodium carbonate, addition of an alkaline solution of 1-amino-3-naphthol-4-sulfonic acid and heating give a red color of an intensity dependent on pH and temperature.[57] This color is readily soluble in ethanol, butanol, amyl alcohol, pyridine, acetone, dioxane, or water. It is insoluble in ethyl ether, petroleum ether, carbon bisulfide, chloroform, or benzene.

Color with chloropicrin in ethanol solution is also suitable for estimation,[58] as is also that with salicylic aldehyde and ferric chloride.[59] Other applicable reagents are *p*-dimethylaminobenzaldehyde,[60] ammonium vanadate,[61] and sodium nitroprusside.[62]

Sample—*Spinal Fluid.* Mix 2 ml. with 4 ml. of water and add 2 ml. of 20 per cent trichloroacetic acid. Mix and centrifuge after 10 minutes. Develop with 1-amino-2-naphthol-4-sulfonic acid.

Serum. Use directly and develop with 1-amino-2-naphthol-4-sulfonic acid.

Urine. Use directly and develop with 1-amino-2-naphthol-4-sulfonic acid.

Procedure—*By β-Naphthoquinone-4-sulfonate.* As reagent dissolve 40 mg. of sodium β-naphthoquinone-4-sulfonate and 250 mg. of sodium sulfite in water and dilute nearly to 200 ml. Add 3 ml. of 6 per cent acetic acid and complete the dilution. Prepare fresh daily.

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[56] C. W. Ballard and P. G. W. Scott, *Chemistry and Industry*, **1952**, 715; Cf. M. Schoog, *Munch. med. Wochschr.* **94**, 2135-7 (1952).

[57] Gianfranco Rapi, *Sperim entile, Sez. chim. biol.* **4**, 11-22 (1953).

[58] Andre Kirshbaum, *Pharm. Acta Helv.* **27**, 229-33 (1952).

[59] Sotosugu Aoki, Ichiro Terai, and Keizo Mori, *Iryo*, **6**, No. 11, 33-5 (1952).

[60] Otto Meyer zu Schwabedissen, *Deut. med. Wochschr.* **78**, 104-5 (1953); M. Smolarek and R. Stahl, *Ibid.* **78**, 273-4 (1953).

[61] O. Wollenberg, *Klin. Wochschr.* **30**, 906-7 (1952).

[62] Henri Laubie, *Bull. soc. pharm. Bordeaux*, **90**, 106-8 (1952).

Prepare tubes containing 3 ml. of sample containing about 0.1 mg. of isonicotinic acid hydrazide, a similar standard, and a reagent blank. To each add 2 ml. of the reagent and then 2 ml. of 8 per cent sodium hydroxide solution. Mix and read at 480 mμ after 15 minutes against a reagent blank.

By 1-chloro-2,4-dinitrobenzene. To 5 ml. of sample in absolute ethanol containing not more than 0.1 mg. of isonicotinic acid hydrazide add 5 ml. of a 5 per cent solution of 1-chloro-2,4-dinitrobenzene in absolute ethanol and about 0.1 gram of borax. Heat at 100° for 10 minutes, cool, and add 25 ml. of methanol. Filter and read at 530 mμ against a reagent blank. Over 0.2 ml. of water must not be present.

By 1-amino-3-naphthol-4-sulfonic acid. As stock reagent dissolve 0.1 gram of sodium metabisulfite in water, add 1 ml. of 10 per cent sodium carbonate solution, and 0.1 gram of 1-amino-2-naphthol-4-sulfonic acid. Dilute to 100 ml. and store in the dark under toluene. For use mix 1 ml. with 2 ml. of water and 1 ml. of 30 per cent sodium carbonate solution.

For color development mix 3 ml. of aqueous sample solution, 1 ml. of 20 per cent trichloroacetic acid solution, and 1 ml. of 30 per cent sodium carbonate solution. Heat for 2-3 minutes at 50° and add 0.2 ml. of the freshly diluted reagent. Extract the color with butanol and read in that solvent at 500 mμ against a blank.

Pyrazamide

Pyrazamide is hydrolyzed to pyrazinoic acid with dilute alkali for estimation. The latter gives an orange-red complex with ferrous ammonium sulfate which is stable for over 2 hours and has a maximum absorption at 460 mμ.[63] The same acid is produced by hydrolysis in the body. The method as given provides for estimation of pyrazamide and pyrazinoic acid separately.

Sample—*Blood.* Mix 1 ml. of blood with 3 ml. of water and add 1 ml. of 10 per cent sodium tungstate solution. Mix, add 2 ml. of 1:9 sulfuric acid, and mix. Decant the supernatant liquid and save. Wash the precipitate with 3 ml. and 3 ml. of water and add these washings to the decantate. Discard the precipitate above. Precipitate sulfate and tungstate with 1 ml. of 10 per cent barium chloride solution and centrifuge.

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[63] William S. Allen, S. M. Aronivic, L. M. Brancone and J. H. Williams. *Anal. Chem.* **25**, 895-7 (1953).

Adjust an aliquot of this extract to pH 7-8 for determination of pyrazinoic acid.

To another aliquot add 1 drop of 50 per cent potassium hydroxide and incubate at 100° for 1 hour. After hydrolysis adjust to pH 7-8 for estimation of the total of pyrazinoic acid and pyrazamide.

Prepare ion-exchange chromotographs in columns 0.5 inch in diameter and 13 inches long, using De Acidite resin treated with hydrochloric acid to convert it to the acid form. Wash the resin well and pack while wet to a depth of 8 inches. Keep the resin covered with water at all times. Pass both samples through such columns. The pyrazoinate ion is sorbed, but extraneous color in the solution passes through. Discard the extracts and wash the columns with water.

Elute the pyrazinoic acid from each column with 30 ml. of 1 per cent sodium chloride solution. Test the eluate with test paper and if not acid adjust to pH 4-7.

Urine. To 10 ml. of urine add 1 ml. of saturated lead acetate solution to precipitate much of the color and extraneous matter. Centrifuge and remove 8 ml. of the supernatant liquid. Add 0.1 ml. of 1:1 sulfuric acid to precipitate lead and centrifuge. Remove an aliquot of 3 ml. and add a drop of 50 per cent potassium hydroxide solution. Incubate at 100° for 1 hour to hydrolyze pyrazamide, adjust the pH to 4-7, and dilute to 10 ml. Adjust another 3 ml. aliquot to pH 4-7 without hydrolysis and dilute to 10 ml.

Procedure—Evaporate an acid solution of pyrazinoic acid to dryness in a 110° oven. Take up in 2 ml. of water and add a few crystals of ferrous ammonium sulfate. Read against distilled water at 460 mμ as pyrazinoic acid. Similarly determine pyrazinoic acid and pyrazamide in the appropriate portion of the sample. Subtract the value for pyrazinoic acid from one for pyrazamide and pyrazinoic acid to obtain pyrazamide.

Pyrazinoic Acid

The method of determination is included with pyrazamide.

Maleic Hydrazide, 1,2-Dihydro-3,4-pyridazinedione

Maleic hydrazide is stable to both strong alkali and strong acid, but ring rupture occurs on reduction to give hydrazine quantitatively.[64]

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[64] Paul R. Wood, *Anal. Chem.* **25**, 1879-83 (1953).

After distillation with special apparatus, this is reacted with *p*-dimethylaminobenzaldehyde to form an azine having a yellow color in dilute solution and red if more concentrated.[65] The color is fully developed within 15 minutes and is stable thereafter. Increased acidity beyond about 0.17 *N*, at which the reagent and azine tend to precipitate, increases the color intensity. Therefore this must be standardized. Slight excess of reagent over the optimum has little effect. Actual recovery approximates 95 per cent of the maleic hydrazide present.

Color increases result from pyrrole, pyrogallol, resorcinol, pyrazoles, and piperidine without reduction. There is increase of color after reduction due to tryptophan and nicotine. No interference occurs with diethanolamine, urea, ethylamine, formaldehyde, ammonium hydroxide, and sodium cyanide. Small amounts of copper do not interfere, but large amounts may.[66] Compounds which break down to sulfur dioxide inhibit color development. Many plant and animal tissues give a pink color which is independent of sample size. A preheating period removes some interferences.

Sample—*Plant and animal tissues.* Grind finely and blend, if necessary adding a known amount of water.

Procedure—Add an aliquot of sample equivalent to 1 gram of sample through a funnel, occasionally more, to the flask of Figure 13.[67] A thick-wall flask is essential because of the corrosion of hot concentrated sodium hydroxide. Wrap the vapor line of the apparatus with asbestos tape to reduce heat loss. A metal valve, as from a rubber suction bulb, is convenient in the tube between the funnel and the flask inlet to prevent steam from blowing back. Wash in with 15 ml. of water and add 30 grams of sodium hydroxide. Place 1 ml. of oxygen-free water and 1 drop of concentrated sulfuric acid in the distillate receiver. Heat the flask on a hot plate until the temperature registered in the thermometer well is 180°. Use silicone oil to conduct the temperature in the thermometer well. Remove the flask from the hot plate and wipe the ball joint dry. Apply silicone grease sparingly so that it will not get into the neck of the flask. Add 15 grams of 30-mesh granular zinc and swirl to distribute it evenly. Clamp the flask in place and start

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65 M. Pesez and A. Petit, *Bull. soc. chim., France,* **1947**, 122-3.

66 L. F. Audrieth and P. H. Mohr, *Ind. Eng. Chem.* **43**, 1774-9 (1951).

67 The apparatus is available from Macalaster Bicknell, 181 Henry Street, New Haven, Conn.

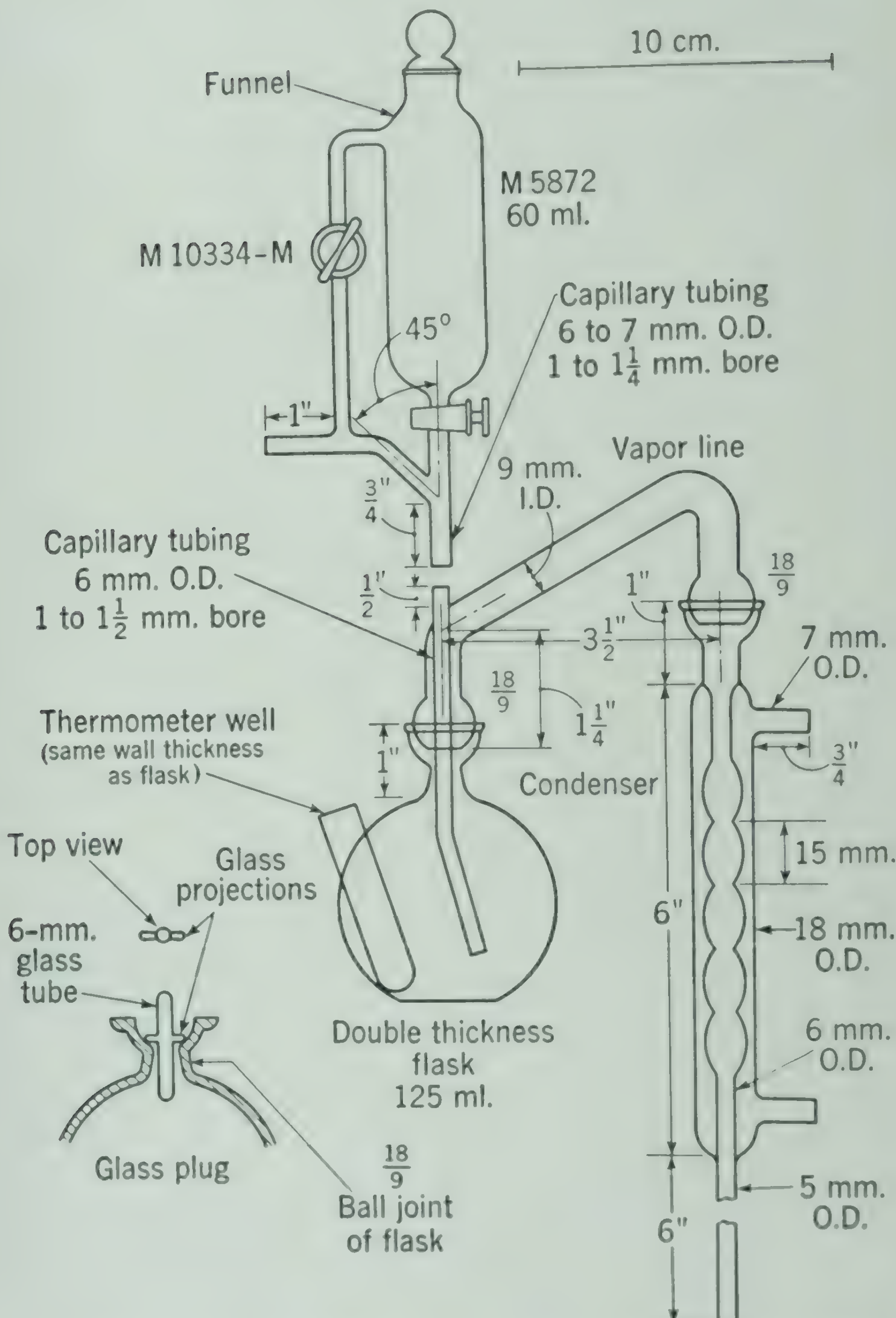

FIG. 13. Reduction-distillation apparatus for determination of maleic hydrazide

the flow of nitrogen to remove oxygen, which might otherwise cause autoxidation of the hydrazine. A bubble rate of 100-150 per minute through the condenser tip is appropriate. If tap water is not available to the condenser at below 10°, use ice water.

While reduction of maleic hydrazide and the corresponding hydrolysis to hydrazine begins at low temperatures, distillation of hydrazine from sodium hydroxide does not begin below 140°. To avoid side reactions provide reheating to that temperature as promptly as possible by heating with a free flame played over the bottom of the flask. Adjust the flow of nitrogen throughout the distillation to maintain the cited bubble rate.

Typical conditions are that the caustic solution starts to boil in not over 1-2 minutes and within 5 minutes the temperature again rises to 180°. The entire distillation should not require over 15 minutes. Check the distillate with pH paper at intervals to be sure it is acid and if necessary add another drop of concentrated sulfuric acid. A preheating 200-ml. mantle at 65-80 volts corresponds closely to the cycle with a free flame.

When the distillation is completed, disconnect at the top of the condenser and wash by sucking the distillate up several times using a rubber suction bulb with an 18/9 ball joint to make the connection. Rinse the condenser with a few ml. of water to keep the distillate at a minimum.

Rinse the distillation flask as soon as practicable or the caustic soda will solidify. Slowly add a small stream of water down the side and when heat of dilution becomes unimportant wash with tap water. Wash with concentrated sulfuric acid before reuse to be sure that no zinc remains unreacted.

If the distillate is believed free of interference, dilute to a known volume and use a 5.5 ml. aliquot. Add 0.5 ml. of fresh reagent containing 0.2 gram of *p*-dimethylaminobenzaldehyde in 5 ml. of 1:17 sulfuric acid. After 15 minutes read at 455 mμ against a reagent blank.

If interference is expected in the distillate, add 1 drop of concentrated sulfuric acid for each 5 ml. Wash successively with 10, 10 and 10 ml. of benzene. After the final extraction, bubble nitrogen through the aqueous acid distillate to remove the last traces of benzene. Removal by heating gives low results. Use a 5 ml. aliquot. Add 0.5 ml. of fresh reagent containing 0.2 gram of *p*-dimethylaminobenzaldehyde in 5 ml. of 1:17 sulfuric acid. Add saturated potassium hydroxide

solution dropwise until the precipitated reagent just persists. Add 1:35 sulfuric acid until this dissolves and dilute to a known volume such as 6 ml.

If the concentration of test substance is unduly low, less than 0.001 mg. in the sample developed, the color will be too faint. Then develop more distillate. Add a saturated solution of potassium hydroxide until precipitation of *p*-dimethylaminobenzalazine is complete. Filter on fritted glass and wash with a few ml. of water. Dissolve from the filter in a micro jar into a receiver. The solvent is 6 ml. of water containing 5-10 drops of concentrated sulfuric acid, barely sufficient to dissolve the precipitate. Correction must be applied for the greater amount of reagent then present.

Pyridine-3-Carboxylic Acid, Nicotinamide, Niacinamide

The corresponding amide has the same vitamin properties as niacin. The characteristic reaction with the pyridine group and cyanogen bromide occurs with both but at a different rate. That is the basis of a method of determining each in the presence of the other given under niacin. As a typical differential, addition of cyanogen bromide followed quickly by aniline produces double the color from niacin as compared with niacinamide.[68] If niacinamide occurs without niacin, it is most satisfactory to saponify because of simplicity of determination thereafter. Using cyanogen bromide and aniline, niacinamide can be determined to 2 per cent, but accumulation of errors may result in 15 per cent error when both are present.[69]

The reaction compound of niacinamide with cyanogen bromide fluoresces while that with niacin does not. Therefore, niacinamide is estimated by that method.[70] A general method for reaction of amides with hydroxylamine hydrochloride to form an acethydroxamic acid is given under amides (page 31).

Samples—*Vitamin mixtures.*[71] Prepare the sample to contain 0.001-0.03 mg. of amide per ml. In the case of vitamin mixtures, capsules, and tablets, heat on a steam bath to effect solution or uniform disper-

[68] Daniel Melnick and Bernard L. Oser, *Ind. Eng. Chem., Anal. Ed.* **15**, 355-6 (1943).

[69] H. Waisman and C. A. Elvehjem, *Ibid.* **13**, 221-4 (1941); Frances W. Lamb, *Ibid.* **15**, 352-5 (1943).

[70] D. K. Chaudhuri and E. Kodicek, *Biochem. J.* **44**, 343-8 (1949); Ramaprasad Banerjee and Sachchidananda Banerjee, *Indian J. Physiol.* **4**, 16-24 (1950).

[71] Frances W. Lamb, *Ind. Eng. Chem., Anal. Ed.* **15**, 352-5 (1943).

sion. Mixtures containing waxes, fats, oils, liver concentrates, etc., are more easily dispersed if 1 ml. of ethylene dichloride is added. Filter the solution to remove oils, fats, waxes, and other insolubles. Discard the first 50-100 ml. of filtrate to avoid loss of amide by sorption on the filter paper. If iron is present in appreciable amounts, precipitate with potassium hydrogen phosphate and filter to avoid a subsequent greenish blue complex. Determine by the differential color development (page 257).

Tissue. Grind 5 grams with sand and about 2 ml. of 1:100 hydrochloric acid. Dilute to 40 ml. and heat in boiling water for 0.5 hour. Cool and add concentrated hydrochloric acid to approximately pH 2. Centrifuge and separate. Wash the residue with 10 ml. of 1:100 hydrochloric acid. Dilute the separated layers to 40 ml. with 1:100 hydro chloric acid. Add 6 ml. of fresh 20 per cent metaphosphoric acid solution and centrifuge. Adjust the clear layer to pH 9.4-9.6, using 30 per cent sodium hydroxide and thymol blue as an external indicator. Heat in boiling water for 0.5 hour and after cooling neutralize with 1:1 hydrochloric acid to pH 7.2 and dilute to 50 ml. Filter for the use of aliquots for fluorimetric estimation.

Procedure—*Fluorimetric.* As sample use an aliquot of the extract containing 0.005-0.25 mg. of niacinamide. To the first sample add 2 ml. of phosphate buffer for pH 7.2 (Vol. I, page 176) and dilute to 14 ml. with water. Prepare the second sample the same way through addition of buffer, but then add 4 ml. of 2.5 per cent cyanogen bromide solution and dilute to 14 ml. Prepare a third sample as an internal standard to contain the same as the second but with 1 ml. of standard containing 0.025 mg. of niacinamide added to this solution. Heat these for 4 minutes at 56-58° and cool in ice water for 5 minutes. Dilute each to 15 ml. To all, or a fraction not less than 5 ml., add 8 ml. of 20 per cent sodium hydroxide solution and dilute to 30 ml. Let stand at room temperature for 0.5 hour and read the fluorescence.

N[1]-Methylniacinamide, N[1]-Methylnicotinamide

This product of metabolism of niacinamide is read fluorimetrically after development by butanol or a ketone.[72] In acid solution it fluoresces

[72] Victor A. Najjar and Robert W. Wood, *Proc. Soc. Exptl. Biol. Med.* **44**, 386-90 (1940); Jesse W. Huff and William A. Perlzweig, *Science* **97**, 538-9 (1943); *J. Biol. Chem.* **150**, 395-400 (1943); R. A. Coulson, P. Ellinger, and M. Holden,

blue; in alkaline solution, green.[73] Trigonelline and niacin do not interfere. There is interference by the coenzymes, diphosphopyridine nucleotide and triphosphopyridine nucleotide. An alternative is to saponify and develop by benzidine.[74] This is accurate to about ±4 per cent. Trigonelline also gives the reaction. Reasonable amounts of urea and glucose do not interfere. If necessary, N^1-methylniacinamide can be sorbed on zeolite and eluted with potassium chloride solution.[75]

Samples—*Urine.* Dilute 1-5 ml. to about 9 ml. and add 0.3 ml. of 45 per cent sodium hydroxide solution. After heating in boiling water for 0.5 hour, cool and dilute to 10 ml. Add 3.3 ml. of a solution of 25 grams of lead nitrate in 95 ml. of water and mix. If not neutral to phenolphthalein, add dry lead nitrate cautiously until it becomes so. Centrifuge, decant, and precipitate the excess lead by addition of dry tripotassium phosphate. Centrifuge and remove a 4-ml. aliquot. Add 16 ml. of ethanol and 5 ml. of 45 per cent sodium hydroxide solution. Heat at 75-80° for 45 minutes, cool, and add 3.7 ml. of concentrated hydrochloric acid. Add 1:1 hydrochloric acid until phenolphthalein is just decolorized, and bring back the color with a drop or two of 2 per cent sodium hydroxide solution. Dilute to 30 ml., mix, and centrifuge. Use an aliquot for color development by benzidine.

For development by a ketone, dilute 2 ml. of urine to 10 ml. with 0.4 ml. of glacial acetic acid and water.

Blood. Add 5 ml. of blood to 6 mg. of dried ammonium oxalate and 4 mg. of potassium oxalate. Lake and precipitate as soon as possible. Add 2 ml. of the oxalated blood dropwise to 8 ml. of 6 per cent trichloroacetic acid. Mix by inversion for 1 minute and after 5 minutes centrifuge. Filter the supernatant layer for development by acetone.

Procedure—*Fluorimetrically by methylethyl ketone.* Prepare flasks for a blank, sample, and sample plus internal standard. Add 2 ml. of sample containing about 0.0025 mg. of test substance per ml. to each sample flask.

To the blank add 4 ml. of water, 0.5 ml. of 1:1 hydrochloric acid,

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Biochem. J. **38**, 150-4 (1944); Nora Levitas, Jean Robinson, Fred Rosen, Jesse W. Huff, and William A. Perlzweig, *J. Biol. Chem.* **167**, 169-75 (1947); Fred Rosen, William A. Perlzweig, and Irwin G. Leder, *Ibid.* **179**, 157-168 (1949); K. J. Carpenter and E. Kodicek, *Biochem. J.* **46**, 421-6 (1950).

[73] Jesse W. Huff, *J. Biol. Chem.* **167**, 151-6 (1947).

[74] Herbert P. Sarett, *Ibid.* **140**, 159-64 (1943).

[75] Victor A. Najjar, *Bull. Johns Hopkins Hosp.* **74**, 392-9 (1944).

and 2 ml. of methylethyl ketone. Heat for 5 minutes on a water bath, add 15 ml. of water and then 4 ml. of 20 per cent monopotassium phosphate. Dilute to 40 ml. with water.

To the sample flasks add 2 ml. of water and to the internal standard 2 ml. of standard containing 0.0025 mg. of test substance per ml. Add 2 ml. of methylethyl ketone and 1 ml. of 20 per cent sodium hydroxide to each. After 5 minutes add 1.5 ml. of 1:1 hydrochloric acid and heat for 5 minutes. Cool and read fluorimetrically.

Fluorimetrically in isobutanol. Add 2 ml. of isobutanol and 0.25 ml. of 15 per cent sodium hydroxide solution to the sample and shake. Separate by centrifuging and read the fluorescence of the isobutanol layer after not less than 5 minutes. Read in terms of a quinine standard previously calibrated.

By acetone. Prepare the sample solution with 0.5 ml. of sample and 0.5 ml. of water, the reagent blank with 1 ml. of water and the standard with 1 ml. of solution containing 0.001 mg. of N^1-nicotinamide per ml. To tubes 1 and 3 add 0.5 mg. of acetone and mix. To each tube add 0.2 ml. of 24 per cent sodium hydroxide solution. Mix and heat in boiling water for 2 minutes. Add 1 ml. of 20 per cent monopotassium phosphate solution to each. Dilute to 10 ml. and read the fluorescence. The result as N^1-methylnicotinamide includes any nucleotide present.

By benzidine. As reagent dissolve 1 gram of benzidine in 25 ml. of ethanol and 75 ml. of 1:15 hydrochloric acid. Add 1 ml. of this to 10 ml. of the prepared sample solution and read 20-40 minutes later against a blank containing everything but the benzidine.

6-Pyridone *of* N^1-Methylniacinamide, 6-Pyridone of N^1-Methylnicotinamide

After ingestion of appreciable amounts of nicotinamide, the 6-pyridone of N^1-methylnicotinamide is excreted in the urine.[76] Its detection consists of 2 steps:[77] removal of pigments and other interfering substances with lead subacetate, and extraction of the pyridone with ether from the filtrate saturated with potassium carbonate.

Procedure—Neutralize an aliquot of the urine to contain 0.2-0.5 mg. of pyridone to pH 6.5-7.5. To decolorize, use 1.3 volumes of saturated

[76] W. Eugene Knox and William I. Grossman, *J. Biol. Chem.* **166**, 391-2 (1946); *Ibid.* **168**, 363-6 (1947).

[77] Fred Rosen, William A. Perlzweig and Irwin G. Leder, *Ibid.* **179**, 157-68 (1949).

lead subacetate solution to 1 volume of urine of specific gravity 1.028. For concentrated urines, a dilution of 1.7:1 will do. If the concentration of pyridone is very low, treat 8 ml. of undiluted urine directly with 250 mg. of solid lead subacetate. Bring the volume to 8 ml. with water, shake briefly, and filter. To a 3-ml. aliquot add 4 grams of potassium carbonate and shake to saturate the solution. Extract the saturated solution with four 10-ml. portions of ether, shaking for 30 seconds with each. Centrifuge and remove the ether layers carefully into a receiver at 40°.

Evaporate the ether in an air current until the residue appears dry. If a noticeable amount of water persists, there is contamination with the aqueous phase and the process must be repeated. To the dry residue add 10 ml. of dry acetone and a pinch of anhydrous sodium sulfate. To a 0.5 ml. aliquot add 0.2 ml. of acetone and 0.01 ml. of 67 per cent potassium hydroxide solution, and cover. Shake mechanically for 1 hour at 23 ± 2°, taking care to avoid overheating. Add 1 ml. of water, shake, and read fluorimetrically against a quinine solution containing 0.02 gamma per ml. Correct for blanks run at the same time.

Pyridine Nucleotides

Nicotinic acid when present in the blood cells is tied up as diphosphopyridine nucleotide or triphosphopyridine nucleotide. Pyridine nucleotides are sorbed from tissue extracts on activated carbon for separation from other cellular components.[78] They are then eluted and read at 340 mμ before and after reduction with hydrosulfite,[79] the value after reduction giving background color. Cyanide also splits the compound for reading at the same wave length.[80]

The method for condensation of N^1-methylnicotinamide with acetone in the presence of alkali gives a fluorescent product with these nucleotides,[81] a product whose fluorescence intensity is about twice as high, per equivalent, as that of the N^1-methylnicotinamide derivative.

[78] Philip Feigelson, J. N. Williams, Jr. and C. A. Elvehjem, *J. Biol. Chem.* **185**, 741-7 (1950).

[79] Otto Warburg and Walter Christian, *Biochem. Z.* **287**, 291-328 (1936).

[80] Sidney P. Colowick, Nathan O. Kaplan, and Margaret B. Ciotti, *J. Biol. Chem.* **191**, 447-59 (1951).

[81] Nora Levitas, Jean Robinson, Fred Rosen, Jesse W. Huff, and William A. Perlzweig, *Ibid.* **167**, 169-75 (1947); Mogens Schon, *Biochem. et Biophys. Acta* **4**, 422-6 (1950).

Sample—*Tissue. Total pyridine nucleotides.* Homogenize approximately 2 grams of frozen tissue for 2 minutes with 10 ml. of 2 per cent trichloroacetic acid and 2 ml. of 30 per cent hydrogen peroxide. This oxidizes any reduced pyridine nucleotide originally present. Allow the homogenate to stand at least 5 minutes at room temperature. Then centrifuge for 5 minutes. Use aliquots of the supernatant liquid for sorption. Grind activated carbon, discard particles which go through a No. 60 sieve, and acid-wash the remaining particles. Each gram will have sorptive capacity for approximately 10 mg. of pyridine nucleotide. Add the acid-washed carbon to a tube over a thin glass-wool plug and cover with another glass-wool plug. Fit the tube with a rubber stopper to permit attachment to a suction flask. Immediately before using the column, rapidly draw 15 ml. of 2 per cent trichloroacetic acid solution through. When the level of the trichloroacetic acid approaches the top of the carbon discontinue the suction and add the solution to be sorbed. Permit the liquid to flow through the column at a rate of about 25 drops per minute. When hydrogen peroxide is used, bubbles of oxygen form and diminish the rate of flow. To correct this apply a slight suction to the receiving flask. After the liquid has passed through, wash with 15 ml. of 2 per cent trichloroacetic acid solution. Draw half of this through with suction and let the rest drip through. Elute with 11 ml. of 10 per cent aqueous pyridine solution for reading in the ultraviolet.

Pyridine nucleotides. Follow the technic for total pyridine nucleotides but in place of the 2 ml. of 30 per cent hydrogen peroxide use 2 ml. more of 2 per cent trichloroacetic acid.

Reduced pyridine nucleotides. The total less the pyridine nucleotides present as such gives the reduced pyridine nucleotides originally present.

Blood. Draw 5 ml. of blood without stasis and add to 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate. Lake the blood and precipitate as soon as possible because the nucleotides are destroyed upon hemolysis.[82] Add dropwise and with shaking 2 ml. of 25 per cent trichloroacetic acid solution and 6 ml. of water. Mix by inversion for 1 minute and allow to stand several minutes. Centrifuge and filter. The filtrate is stable for several days in the refrigerator. Develop with acetone and alkali.

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[82] Henry I. Kohn and J. Raymond Klein, *J. Biol. Chem.* **135**, 685-9 (1940).

Procedure—*In the ultraviolet.* To 5 ml. of eluate add 1 ml. of 4.2 per cent sodium bicarbonate solution and 1 ml. of water. To another 5 ml. of eluate add 0.4 ml. of water and 0.6 ml. of a 1.8 per cent solution of sodium bicarbonate. Place both tubes in boiling water for 90 seconds. Place in an ice bath and add 1 ml. of 5.3 per cent sodium carbonate solution. Bubble oxygen through one tube for 2.5 minutes and read both at 340 mμ against water. The difference in reading between oxidized and reduced samples is due to reduced protein nucleotides present.

By acetone and alkali. To 1 ml. of sample add 0.5 ml. of acetone and mix. Then add 0.2 ml. of 24 per cent sodium hydroxide solution and mix immediately. After 15 minutes add 0.3 ml. of 1:1 hydrochloric acid solution and mix at once. Immerse in boiling water for 2 minutes and add 1 ml. of 20 per cent monobasic potassium phosphate solution. Dilute to 10 ml. and mix. The fluorescence reaches a maximum immediately.

N,N-Diethylniacinamide, Nicetamide

Determine with cyanogen bromide and aniline (page 257) but cool and read at 436 mμ.[83]

2-Methyl-3-hydroxy-4,5-di(hydroxymethylpyridine), Pyridoxine, Vitamin B_6

The phenolic nature of pyridoxine, or vitamin B_6, permits coupling with several reagents to obtain a color reaction. The most specific is that with 2,6-dichloroquinonechloroimide[84] in a strongly buffered alcoholic solution to give a blue color.[85] 2,6-Dibromoquinonechloroimide can be substituted.[86] Technics are applied in which sorption and elution are followed by development in a 2-phase system,[87] sometimes with

[83] Olle Wallén, *Farm. Rev.* 46, 233-6 (1947).

[84] H. D. Gibbs. *J. Biol. Chem.* 72, 649-64 (1927); John V. Scudi, W. A. Bastedo, and T. J. Webb, *Ibid.* 136, 399-406 (1940).

[85] Eric T. Stiller, John C. Keresztesy, and Joseph R. Stevens, *J. Am. Chem. Soc.* 61, 1237-42 (1939); John V. Scudi, *J. Biol. Chem.* 139, 707-20 (1941); Melvin Hochberg, Daniel Melnick and Bernard L. Oser, *Ibid.* 155, 109-17 (1944).

[86] Teodor Canbäck and Maj Lis Lindholm, *Finska Kemistsamfundets Medd.* 54, 134-40 (1945).

[87] John V. Scudi, H. T. Koones, and J. C. Keresztesy, *Proc. Soc. Exptl. Biol. Med.* 43, 118-22 (1940); John V. Scudi, Klaus Unna, and William Antopol, *J. Biol. Chem.* 135, 371-6 (1940); M. Swaminathan, *Nature*, 145, 780 (1940); *Indian J.*

the sorbant still present. Single phase development is preferable. It is necessary to control to avoid interference by color with phenols, amines, and other structures. Rather than attempt to isolate the pyridoxine, that compound is rendered inactive by the addition of excess borate to a blank prepared from the sample with a strongly alkaline buffer. Ascorbic acid interferes but is oxidized by shaking the alkaline extract with manganese dioxide. Interference by histidine and histamine can be prevented by their precipitation with mercury salts (page 000). Pyridoxine is completely sorbed on Permutit in a 0.01 *N* buffer for pH 5 and eluted by hot potassium chloride-hydrochloric acid solution.[88] Ultraviolet light destroys the vitamin after exposure,[89] but normal laboratory daylight does not. The pH to which the solution is buffered affects the rate of color development. When fully developed the solutions conform to Beer's law. Results show an average deviation of ±1.1 per cent.

The intensity is affected by the variable concentration of reagent due to reaction with other substances in the blank. Therefore, standards must be prepared by known additions to the same menstruum, alike in salinity and other properties. Bound pyridoxine must first be hydrolyzed by heating with strong acid,[90] and that found in vegetable products is usually esterified. The method does not determine the related biologically-active compounds, pyridoxal and pyridoxamine.[91]

Diazotized sulfanilic acid gives a yellow to orange color with pyridoxine.[92] This differentiates it from bright yellow with pyridoxal and pinkish with pyridoxamine.[93] Major interferences must be removed.[94] Then the solution is read fluorometrically.

Med. Res. **29**, 561-6 (1941); John V. Scudi, *J. Biol. Chem.* **139**, 707-20 (1941); O. D. Bird, J. M. Vandenbelt, and A. D. Emmett, *Ibid.* **142**, 317-22 (1942); Albert F. Bina, James M. Thomas, and Elmer B. Brown, *Ibid.* **148**, 111-6 (1943).

88 Ryn Ping Kun, *Kitasato Arch. Exptl. Med.* **24**, No. 2, 77 (1951).

89 Melvin Hochberg, Daniel Melnick, and Bernard L. Oser, *J. Biol. Chem.* **155**, 129-36 (1944); Melvin Hochberg, Daniel Melnick, Louis Siegel, and Bernard L. Oser, *Ibid.* **148**, 253-4 (1943).

90 John V. Scudi, *Ibid.* **139**, 707-20 (1941); *Ibid.* **145**, 637-9 (1942); Louis Siegel, Daniel Melnick, and Bernard L. Oser, *Ibid.* **149**, 361-7 (1943).

91 Esmond E. Snell, Beverly M. Guirard, and Roger J. Williams, *Ibid.* **143**, 519-30 (1942); Esmond E. Snell, *Ibid.* **154**, 313-4 (1944); Stanton A. Harris, Dorothea Heyl, and Karl Folkers, *J. Am. Chem. Soc.* **66**, 2088-92 (1944).

92 M. Swaminathan, *Nature,* **145**, 780 (1940).

93 A. A. Ormsby, A. Fisher, and F. Schlenk, *Arch. Biochem.* **12**, 79-81 (1947).

94 Albert F. Bina, James M. Thomas and Elmer B. Brown, *J. Biol. Chem.* **148**, 111-16 (1943).

Other methods include development with phosphotungstic-phosphomolybdic acid,[95] diazotized *p*-aminoacetophenone,[96] and 29 per cent ferric chloride.[97] The reaction of cyanogen bromide with niacin (page 247 *et seq.*) is also given by pyridoxine under the influence of heat.[98]

Rupture of the pyridine ring,[99] commonly applied to nicotinic acid, is also applicable by coupling with an aromatic complex.[100] Compounds such as *α*-picoline, *α*-aminopyridine, and picolinic acid give negligible color by this reaction.

Samples—*Biological materials.* Hydrolyze a sample containing 0.03-0.2 mg. of pyridoxine by adding 10 ml. of 1:2 hydrochloric acid and heating in boiling water for 1 hour with occasional stirring. For material of low potency where more than 3 grams is required increase the amount of acid proportionally. For liquid samples use stronger acid to allow for the aqueous dilution. Cool and adjust the pH to 3 with 4 per cent sodium hydroxide solution and an outside indicator. Add 3 ml. of buffer solution containing 7.3 per cent of disodium phosphate dihydrate and 16.7 per cent of citric acid. For sorption add 2.5 grams of Lloyd's reagent. Stopper and shake occasionally for 5 minutes. Centrifuge and discard the supernatant liquid. Wash the residue with 15 ml. of 1:10,000 hydrochloric acid. Centrifuge and discard the washing. Add 5 ml. of 8 per cent sodium hydroxide solution to elute the pyridoxine, dilute to 20 ml. with water, and invert several times. Centrifuge and pipet out a 10-ml. aliquot into another tube. Add 50 ml. of isopropanol, mix, and centrifuge. Decant the clear solution and adjust the pH to 5-7 with a few drops of concentrated hydrochloric acid, for development with 2,6-dichloroquinonechloroimide.

Pharmaceuticals, ascorbic acid absent. Treat as for biological material.

Pharmaceuticals containing ascorbic acid. Blend enough tablets to give 2-6 mg. of pyridoxine with 100 ml. of water and 8.3 ml. of 1:6

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[95] Richard Kuhn and Irmentraut Löw, *Ber.* **72B**, 1453-7 (1939); M. Swaminathan, *Nature* **145**, 780 (1940).

[96] Elmer B. Brown, Albert F. Bina, and James M. Thomas, *J. Biol. Chem.* **158**, 455-61 (1945).

[97] Gilberto Guimarães Villela, *Anais. assoc. quím. Brasil* **7**, 168-70 (1948); R. D. Greene, *J. Biol. Chem.* **130**, 513-8 (1939).

[98] Arthur E. Teeri, *Chemist-Analyst* **41**, 62-4 (1952).

[99] W. König, *J. prakt. Chem.* **69**, 105 (1904).

[100] James P. Sweeney and Wallace L. Hall, *J. Assoc. Official Agr. Chemists* **35**, 479-83 (1952).

sulfuric acid for 2 minutes. Add a few drops of caprylic alcohol to prevent foaming and dilute to not over 200 ml. Heat for 20 minutes on a steam bath and cool. Dilute to 250 ml. and use a 15-ml. aliquot. Add 5 ml. of 8 per cent sodium hydroxide solution and 0.2 gram of manganese dioxide to destroy ascorbic acid. Shake for 5 minutes. Add a 5-ml. aliquot of the suspension to 25 ml. of isopropanol and centrifuge. Use an aliquot of the supernatant liquid for the assay with 2,6-dichloroquinonechloroimide.

Rice bran.[101] Place a 0.5-gram sample in 5 ml. of water and precipitate at pH 8 with 0.5 ml. of clear, saturated basic lead acetate solution. Remove the precipitate, wash with water, and, using 1:3 hydrochloric acid, adjust the pH of the combined supernatant liquids to 3. Dilute the solution to 20 ml. and allow 10 ml. to percolate at a rate of 0.5 ml. per minute through a glass column containing 100 mg. of Superfitrol diluted with 900 mg. of Decalso. Prepare a buffer for pH 3 containing 6 ml. of 1:11 hydrochloric acid and 4 ml. of 2.1 per cent citric acid in 0.8 per cent sodium hydroxide solution. Wash the sorbent 3 times with 5-ml. portions of water which contained 1/8 volume of the buffer. Elute the vitamin with three 5-ml. portions of 1 per cent sodium hydroxide solution. Develop an aliquot with 2,6-dichloroquinonechloroimide.

Foods. Blend 1-5 grams of sample with 20 ml. of 1:900 sulfuric acid solution. Autoclave for 30 minutes at 15 pounds pressure, cool, and add 5 ml. of an acetate buffer solution for pH 4.3 containing 0.2 gram each of takadiastase and papain. Incubate the whole at 40° for 2 hours and centrifuge for 5 minutes. Decant the extract, wash the residue with about 20 ml. of water, and centrifuge. Adjust the combined extract and washings to pH 7 with 30 per cent sodium hydroxide solution and dilute to 100 ml.

To an aliquot of about 35 ml. of this extract add 2 ml. of 25 per cent sodium tungstate solution. Follow this with 0.5 ml. of concentrated sulfuric acid. Mix by inverting and let stand for 5 minutes. Centrifuge 2-3 minutes, decant the extract, and wash the precipitate with 5 ml. of water. Add the washing to the extract. Disregard slight turbidity at this stage. Adjust the pH to 3 with 30 per cent sodium hydroxide solution by a glass electrode. If there is precipitation, remove by centrifuging. To the solution, add 0.5 gram of Superfiltrol, mix, and allow to stand for 30 minutes. Invert the tube at intervals, centrifuge, and

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101 John V. Scudi, *J. Biol. Chem.*, **145**, 637-9 (1942).

discard the extract. Wash the Superfiltrol, which now contains the vitamin B_6, twice with 15 ml. of a buffer containing 5.44 per cent of acetic acid and 11.1 per cent of anhydrous sodium acetate and discard the washings. Decant the last washing as completely as possible.

Elute the vitamin B_6 from the Superfiltrol by adding to it 20 ml. of 0.5 per cent sodium hydroxide in ethanol. Shake and place in a water bath at 60-65° for 30 minutes. Twirl occasionally. Cool, centrifuge, and decant the eluate. Wash the Superfiltrol with another 5 ml. of alkaline ethanol solution and combine the washings. Adjust the pH of the solution to 7.3 with 12 per cent acetic acid by means of the glass electrode. Dilute with ethanol to the desired volume and filter. Use 10-ml. aliquots containing 0.01-0.02 mg. for fluorescent reading with diazotized sulfanilic acid.

Procedure—*By 2,6-dichloroquinonechloromide.* Purify the solid reagent by fractional precipitation from 2 per cent solution in acetone by addition of water. Store the crystals in a refrigerator after air-drying by suction. For use prepare a 0.02 per cent solution in isopropanol, store under refrigeration, and discard when it turns pink.

To 6 ml. of sample ssolution containing 0.005-0.05 mg. of pyridoxine and 5 ml. of isopropanol, add 2 ml. of a solution containing 160 grams of ammonium chloride and 160 ml. of concentrated ammonium hydroxide per liter and 1 ml. of 5 per cent boric acid solution. In a second tube replace the boric acid solution with water. A third tube replaces the boric acid with 1 ml. of solution containing 0.01 mg. of pyridoxine in 1:120 hydrochloric acid. Add 1 ml. of reagent to the first tube, mix, and after 1 minute use this to set the zero point at 620 mμ. Similarly treat the second and third tubes and read after 1 minute. Calculate from the standard or read against a standard curve prepared by addition of pyridoxine to substantially the same menstruum.

Fluorescence by diazotized sulfanilic acid. As reagent, pipet 2.5 ml. of a 0.32 per cent solution of sulfanilic acid in 91:9 sulfuric acid into a brown 25-ml. glass-stoppered graduate and place in an ice bath. After 5 minutes add 0.4 ml. of a 10 per cent sodium nitrite solution, mix, and dilute to 10 ml. with water. Make up the solution before each use and keep in the ice bath.

Add 4 ml. of 50 per cent sodium acetate solution and 2 ml. of water to a 10 ml. aliquot of the sample containing 0.01-0.02 mg. of pyridoxine and mix. Follow by 1 ml. of diazotized reagent and mix. Add 2 ml. of 5.5 per cent sodium carbonate solution and mix. Read the fluor-

escence of the yellow solution against water using blue and yellow filters. The fluorescence fades only slowly. Subtract the reading on a blank in which the diazo reagent was substituted by water.

3,3-Diethyl-2,4-diketotetrahydropyridine, Ketotetrahydropyridine, Presidon

Presidon is estimated in urine by its fluorescence in alcoholic solution before and after quenching with hydroxylamine.[102] The method is subject to error due to fluorescent substances present in pathologcal urines. Also, hydroxylamine can quench appreciable amounts of interfering fluorescence so that high apparent values are obtained. To avoid these difficulties extract Presidon from urine with ether.

Procedure—Add an amount of urine containing about 0.005 mg. of Presidon and 1 ml. of saturated sodium tetraborate solution to about 23 ml. of 25 per cent aqueous potassium chloride solution. Extract 3 times with 25-ml. portions of ether, shaking vigorously each time for 2 minutes. If an emulsion forms, break it by centrifuging. Combine the extracts, add 1 ml. of 1 per cent aqueous ascorbic acid solution, and boil off the ether on a steam bath. Remove the last traces of ether with a stream of nitrogen and avoid overheating the residue. Dilute with water to get a concentration of approximately 0.001 mg. of Presidon per ml.

Mix 5-ml. aliquots of these dilute extracts and 10 ml. of ethanol. To one add 1 ml. of water and to the other 1 ml. of 10 per cent aqueous hydroxylamine hydrochloride. Add 1 ml. of 10 per cent sodium hydroxide solution to each tube. Mix well. Heat the sample containing hydroxylamine at 75-80° for 20 minutes. Cool to room temperature in cold water and take fluorescence readings with 370 mμ and 460 mμ filters as used for thiochrome.

Antihistamines

The reactions of the antihistamines are so similar that many of them are concentrated here. Specific methods for some structures then follow. The commercially available compounds in general give an in-

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102 U. Kubli and E. Schmid, *Helv. Chim. Acta* 28, 213-20 (1945); Erich Hirschberg, Martha Fine Greenberg, Elmer De Ritter, and Saul H. Rubin, *J. Am. Pharm. Assoc.* 37, 288-91 (1948); Elmer De Ritter, Fred W. Jahns, and Saul H. Rubin, *ibid.* 38, 319-21 (1949).

soluble reineckate which can be dissolved in acetone for reading.[103] The following tabulation gives first the chemical name of the free base followed by some of the commercial names used for the product, frequently as a designated salt.

2-(N-benzylanilinomethyl)imidazoline
  Antistine hydrochloride
2-(benzhydryloxy)-N,N-dimethylethylamine
  Diphenhydramine hydrochloride
  Benadryl
  Amidryl
2-[(5-chloro-2-thenyl)(2-dimethylaminoethyl)amino]pyridine
  Chlorothenylpyramine hydrochloride
  Tagathen hydrochloride
  Chlorothen hydrochloride
2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl]pyridine
  Doxylamine succinate
  Decapryn succinate
2-[Benzyl(2-dimethylaminoethyl)amino]pyridine
  Tripelennamine hydrochloride or citrate
  Pyribenzamine hydrochloride or citrate
2-[α-(2-dimethylaminoethyl)benzyl]pyridine
  Prophenpyridamine maleate
  Trimeton
  Inhiston
2-[(2-dimethylaminoethyl)(p-methoxybenzyl)amino]pyridine
  Pyranisamine maleate
  Neo-Antergan maleate
  Pyrilamine maleate
2-[(2-dimethylaminoethyl)(p-methoxybenzyl)amino]pyrimidine
  Thonzylamine hydrochloride
  Neohetramine hydrochloride
  Anahist
  Resistab
2-[(2-dimethylaminoethyl)-2-thenylamino]pyridine
  Methapyrilene hydrochloride
  Thenylene hydrochloride

[103] F. J. Bandelin, E. D. Slifer, and R. E. Pankratz, *J. Am. Pharm. Assoc.*, 39, 277-80 (1950).

Histadyl hydrochloride
Thenylpyramine hydrochloride
2-methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene
Phenindamine tartrate
Thephorin tartrate

An alternative [104] is to open up the pyridine ring with cyanogen bromide and couple with aniline.

All such compounds have characteristic absorption spectra in the ultraviolet with well-defined maxima.[105] A table is given under procedure. The peak read is that due to the ethylenediamine group in acid solution. The use of ultraviolet light is preferable to methods of color development because there is no source of experimental error. In this case ultraviolet absorption is more sensitive than many colorimetric procedures, for as little as 4 mg. of thenylpyramine hydrochloride per ml. can be determined with an accuracy of ±0.5 per cent. Those which have a nitrogen joined to the second carbon of the pyridine ring can also be read fluorophotometrically.[106]

Sample—*Tablets.* Crush 20 tablets and digest an aliquot equivalent to 50 mg. of test substance on a steam bath with 50 ml. of 1:100 sulfuric acid for 30 minutes. Cool, filter, and wash the paper with 1:100 sulfuric acid to make to 100 ml. Use an aliquot for precipitation as the reineckate.

Capsules. Mix the contents of 20 capsules. Complete as for tablets from ". . . digest an aliquot . . . ."

Ointments. Dissolve or suspend an aliquot equivalent to 50 mg. of test substance in 50 ml. of petroleum ether. Extract with four 10-ml. portions of 1:100 sulfuric acid. Extract the combined extracts with 5 ml. of petroleum ether and discard the extract. Filter the acid solution through cotton wet with 1:100 sulfuric acid. Dilute to 50 ml. with the same acid. Use an aliquot for precipitation as the reineckate.

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104 Hurd M. Jones and Edward S. Brady, *J. Am. Pharm. Assoc., Sci. Ed.* **38**, 579-80 (1949).

105 R. C. Clapp, J. H. Clark, J. R. Vaughan, J. P. English and G. W. Anderson, *J. Am. Chem. Soc.* **69**, 1549 (1947); Eric W. Martin and Joseph W. E. Harrisson, *J. Am. Pharm. Assoc., Sci. Ed.*, **39**, 390-2 (1950); T. V. Parke, A. M. Tibley, E. E. Kenedy, and W.W. Hilty, *Anal. Chem.* 23, 953-7 (1951); Daniel Banes, *J. Assoc. Official Agr. Chemists* **34**, 703-10 (1951).

106 Ely Perlman, *J. Pharmacol. Exptl. Therap.* **95**, 465-81 (1949).

Tablets containing antipyretic compounds.[107] The technic is developed for 0.33 gram of methapyrilene hydrochloride, 3.5 grams of acetylsalicylic acid, 2.5 grams of acetophenetidin, and 0.5 gram of caffeine. Grind 20 tablets and suspend about 1.5 grams in 25 ml. of 1:9 hydrochloric acid. Extract with 40 ml. and four 20-ml. portions of ether. Wash the combined ether extracts with 10 ml. of 1:9 hydrochloric acid and then with 10 ml. of water. Wash the acid and water extracts with 20 ml. and 20 ml. of ether. Add the aqueous and acid solutions to the previous acid extract as sample for determination of caffeine and the antihistamine. Combine the ether extracts for estimation of acetylsalicylic acid and acetophenetidin. Wash the ether extracts with 25 ml. of 5 per cent sodium bicarbonate solution and then with 15 ml. of water. Wash these extracts with 20 ml. and 20 ml. of ether. Combine the alkaline and aqueous extracts as samples for determination of acetylsalicylic acid. Add the ether extracts to the previous ether extracts for determination of acetophenetidin.

Extract the previous aqueous phase containing caffeine and antihistamine with four 30-ml. portions of chloroform. Wash these consecutively with a 5-ml. portion of 1:6 hydrochloric acid. Determine caffeine in these chloroform extracts. Dilute the combined aqueous extracts to 100 ml. for estimation of the antihistamine as the reineckate.

Solutions. Make 40 ml. of solution alkaline with 5 ml. of 4 per cent sodium hydroxide solution Extract with three successive 40-ml. portions of ether. Discard the alkaline layer and wash the three ether extracts successively with 20 ml. and 20 ml. of 0.4 per cent sodium hydroxide solution and then with 20 ml. of water. Extract the antihistamine from the ether solutions successively with 20 ml., 20 ml., and 5 ml. of 1:360 sulfuric acid. Dilute the combined acid extracts to 50 ml. with the same acid. Dilute an aliquot equivalent to 6.25 mg. of diphenhydramine hydrochloride or 0.625 mg. of the others listed for reading in the ultraviolet, to 25 ml. with 1:360 sulfuric acid.

Alternatively, acidify 25 ml. of solution with 1 ml. of 1:1 sulfuric acid and wash with three successive 40-ml. portions of ether. Wash the ether solutions successively with 15 ml. of water. Add these washings to the washed acid extract and dilute to 50 ml.

Make an aliquot alkaline with 4 per cent sodium hydroxide solution, dilute to 30 ml., and extract with 30 ml. of chloroform. Wash the chloroform extract with 10 ml. of water and filter through cotton.

[107] Kenneth E. Holt, *Bull. Natl. Formulary Comm.* **13**, 121-3 (1945).

Repeat the extraction with three 20-ml. portions of chloroform. Dilute the combined extracts to 100 ml. with chloroform. Extract an aliquot of the chloroform extract containing about 2.5 mg. of diphenhydramine hydrochloride or 0.25 mg. of the other compounds with 10 ml. of 1:360 sulfuric acid, and read the solution in the ultraviolet.

Procedure—*As the reineckate.* To a sample solution containing 2-10 mg. of the test substance add 10 ml. of 1:99 sulfuric acid. Chill in an ice bath and add 5 ml. of fresh saturated aqueous ammonium reineckate dropwise while swirling. After 1 hour in the bath, filter by suction in a sintered glass crucible. Wash with 5 ml. and 5 ml. of water at 5°. Draw air through. Dissolve from the crucible with acetone and dilute to 25 ml. with acetone. Read at 525 mμ against acetone, using a curve prepared with the test substance.

By cyanogen bromide. Mix a 3-ml. sample containing 0.2-1 mg. of test substance per ml. with 1 ml. of 4 per cent cyanogen bromide reagent and 5 ml. of 2 per cent potassium acid phthalate solution as buffer. Let stand for 15 minutes and add 1 ml. of a 4 per cent solution of aniline in ethanol. After 20 minutes read at 400-460 mμ against a water blank.

In the ultraviolet. Data are given in Table 13 which follows.

TABLE 13. WAVE LENGTHS FOR READING VARIOUS ANTIHISTAMINES

(More detailed names for many of the compounds appear elsewhere)

Chemical Name	*Maximum(mμ)*	$E^{1\%}_{1\,cm.}$
Thonzylamine hydrochloride	313	104
Methapyrilene hydrochloride	315	269
Pyranisamine maleate	314	196
Tripelennamine hydrochloride	314	274
Prophenpyridamine maleate	265	212
Chloroprophenpyridamine maleate	264	219
Doxylamine succinate	262	227
Diphenhydramine hydrochloride	258	16.5

N′-*p*-METHOXYBENZYL-N′-2-PYRIDYL-N′-DIMETHYL ETHYLENEDIAMINE, NEOHETRAMINE

Neohetramine gives a maximum color absorption at 510 mμ, after heating with 2-thiobarbituric acid.[108] The reaction is specific for the

[108] Milton Feldstein and Niels C. Klendshoj, *J. Am. Pharm. Assoc.* **40**, 370-2 (1951).

pyrimidine group with substituents in the 2-position and none in the 4-, 5-, and 6-positions. The color is stable for 15 minutes. The minimum amount of neohetramine which can be determined is 0.01 mg. in a 5-ml. hydrochloric acid extract. The method is accurate to about ±2-5 per cent, depending on the size of sample.

Sample—*Tissue*. Place 50 grams of tissue containing 0.01-0.06 mg. of neohetramine into a blender, with 2 ml. of concentrated ammonia and 200 ml. of ethylene dichloride. Homogenize for 1-2 minutes and let stand until the layers separate. Filter the solvent layer and extract 100 ml. with 10 ml. of 1:120 hydrochloric acid. Centrifuge to remove small droplets of solvent and use the acid extract as sample.

Blood. To 5 ml. of oxalated blood in a separatory funnel, add 10 ml. of water and 2 ml. of concentrated ammonium hydroxide. Rotate to hemolyze the blood, extract with 100 ml. of carbon tetrachloride, and wash the solvent layer with 50 ml. of water. Continue as for tissue from "Filter the solvent layer."

Urine. Adjust 50 ml. of urine to pH 7.8 by addition of 2 grams of anhydrous disodium phosphate. After the salt has dissolved, extract with 100 ml. of carbon tetrachloride. Wash the solvent layer with 50 ml. of water. Continue as for tissue from "Filter the solvent layer."

Tablets. Powder in a mortar and add 1:120 hydrochloric acid. Dilute to 500 ml. with the same acid. Filter and extract a 50-ml. aliquot with 100 ml. of carbon tetrachloride. Wash the solvent layer with 50 ml. of water. Continue as for tissue from "Filter the solvent layer."

Procedure—To a 5-ml. aliquot of hydrochloric-acid extract add 2 ml. of a saturated solution of 2-thiobarbituric acid in 1:10 hydrochloric acid and heat at 110-130° C. Continue to heat for an additional two hours after the solution has evaporated to dryness. Let cool, add 10 ml. of 1:9 ammonium hydroxide, and stir to insure complete solution. Read within 10 minutes at 510 mμ against a water blank.

2-*p*-Chlorophenylguanidino-4-*p*-diethylamino-6-methylpyrimidine dihydrochloride, Compound 3349

Compound 3349, an antimalarial, is precipitated with potassium mercuri-iodide and determined turbidimetrically with an accuracy of ±10 per cent for amounts down to about 0.002 mg.[109] Many basic

[109] A. Spinks, *Ann. Trop. Med. Parasitol.* 39, 182-9 (1945).

drugs including mepacrine and quinine interfere, but normal blood, urine, and tissues contain no reacting bases. This method is not suitable for determination of the small amounts found in human blood.

A more sensitive method is by hydrolysis of the base under pressure to *p*-chloroaniline, followed by diazotization and coupling to give a red azo dye. This is applied to any biological material for determination of about 0.0004 mg.[110] Recovery from blood is accurate to about ±9 per cent, from plasma to about ±5 per cent. Quinine does not interfere, nor mepacrine except at high concentrations. Phenacetin and procaine interfere. Normal plasma, spleen, lung, kidney, and urine contain no reacting bases, but blanks up to 0.001 mg. per 100 ml. are found for whole blood. Paludrin gives the same reaction.

Sample—*Blood. High drug concentration.* Dilute 10 ml. with 20 ml. of water, add 5 ml. of 10 per cent sodium hydroxide solution, mix, and extract continuously with ether for 12 hours. Continue as for urine, starting with "Remove the solvent and dry at 100°." Dilute the final hydrochloric-acid extract to 5 ml. instead of 10 ml.

Blood. Low drug concentration. Dilute 5 ml. with an equal volume of water and add half its volume of 40 per cent sodium hydroxide solution. Warm at 50° for 30 minutes and let cool. Add 20 ml. of benzene containing 2 per cent of absolute ethanol. Shake for 5 minutes. Centrifuge until the emulsion has cleared. Freezing and recentrifuging are sometimes necessary. Mix as large an aliquot as possible with 1.3 ml. of 1:120 hydrochloric acid and shake for 3 minutes. Centrifuge, transfer the aqueous layer to an ampoule, seal this, and autoclave at 20-25 pounds per square inch for 12 hours. Use a 1-ml. aliquot for diazotization.

Plasma. Low drug concentration. Centrifuge a sample of blood for 20 minutes to give 10 ml. of plasma. Decant the plasma and recentrifuge for 20 minutes. Treat 5 ml. of plasma, free of leucocytes, with 0.25 volume of 40 per cent sodium hydroxide solution, warm at 50° for 30 minutes, and shake for 5 minutes with 10 ml. of benzene containing 2 per cent of absolute ethanol. Continue as for blood, starting with "Centrifuge until the emulsion has cleared."

Tissue. High drug concentration. Homogenize 2 grams of tissue in 20 ml. of water and 5 ml. of 10 per cent sodium hydroxide solution.

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[110] A. Spinks and Mary M. Tottey, *Ibid.*, 39, 190-6 (1945).

Extract continuously with ether for 12 hours. Continue as for urine, starting with "Remove the solvent and dry at 100°."

Tissue. Low drug concentration. Homogenize 1 gram of tissue in water and dilute to 10 ml. Treat 5 ml. as with urine.

Urine. High drug concentration. Treat 5-50 ml. of sample solution with 1 volume of a 10 per cent solution of sodium hydroxide and extract automatically with ether for 6 hours. Remove the solvent and dry at 100°. Dissolve the bases by warming for 3 minutes, with shaking, with 2 ml. of 1:120 hydrochloric acid. Repeat the acid extraction twice and dilute the combined extracts to 10 ml. with 1:120 hydrochloric acid. If cloudy, centrifuge or filter through glass wool. Use for turbidimetric determination.

Urine. Low drug concentration. Treat as for plasma except to extract the benzene solution of base with 2 ml. of 1:120 hydrochloric acid instead of with 1.3 ml.

Bile. Low drug concentration. Treat the same as with urine.

Feces. Low drug concentration. Ball-mill 10 grams in 50 ml. of 1:20 hydrochloric acid and dilute to 500 ml. Treat 5 ml. as with urine.

Procedure—*High drug concentration. Turbidimetric.* As reagent dissolve 1.36 grams of mercuric chloride in 25 ml. of water, and 3.32 grams of potassium iodide in 25 ml. of water. Add the first solution to the second slowly with stirring and dilute to 100 ml. Filter and store in a dark glass-stoppered bottle. To 2 ml. of sample add 0.2 ml. of potassium mercuri-iodide reagent and read immediately against a standard containing 1 mg. per ml. of drug.

By diazotization. To 1 ml. of hydrolyzed sample add 0.1 ml. of 15 per cent sodium hydroxide solution. Add 0.1 ml. of a 0.1 per cent solution of sodium nitrite. After 15 minutes add 0.2 ml. of a 1 per cent solution of N-β-sulfatoethyl-*m*-toluidine and 0.3 ml. of a 30 per cent solution of sodium acetate. After 10 minutes add 4 drops of concentrated hydrochloric acid, dilute to 2 ml., and read within an hour against an appropriate blank at 510 mμ.

1-(*p*-Chlorophenyl)-5-isopropylbiguanide, Paludrine, Drinupal

A simple method of determination of Paludrine is by extraction with benzene and coupling with bromothymol blue.[111] Several of the

[111] Earl J. King, I. D. P. Wooton, and Margaret Gilchrist, *Lancet* 250, 886-7 (1946); J. C. Gage and F. L. Rose, *Ann. Trop. Med. Parasitol.* 40, 333-6 (1946).

same group of compounds can be estimated by this method. It is applicable for clinical use for determination of 0.001-0.002 mg. per ml.

Another method is by formation of a copper complex in which 2 molecules of Paludrin associate with 1 atom of copper, followed by determination of copper with sodium diethyldithiocarbamate. The color is given by mono- and dialkyl-substituted *p*-chlorophenylbiguanides but not by their presumed metabolic breakdown products. This method is suitable for determination of 0.5-10 mg. of Paludrin per 1000 ml. and is accurate to about ±3 per cent. The method by diazotization given for compound 3349 (page 284) is also applicable to Pauldrin.

Sample—*Blood.* Proceed as for compound 3349, for "Blood. Low drug concentration" (page 283) through "Centrifuge until the emulsion has cleared." Use 5 ml. of blood and 25 ml. of benzene-2 per cent ethanol reagent. Use the clear solvent extract for color development with bromothymol blue.

Urine. To 2 ml. of urine add 1 ml. of a solution containing 0.5 per cent of copper sulfate pentahydrate and 1.33 per cent of ammonium chloride. Add 1 ml. of 4 per cent sodium hydroxide solution, mix, and let stand a few minutes. Extract with 5 ml. of benzene. Separate the benzene layer, wash with 1 ml. of water, and use the benzene extract for determination as the copper complex.

Procedure—*By bromothymol blue.* Prepare the reagent in a phosphate buffer solution at pH 7 as follows: Dissolve 40 mg. of bromothymol blue by heating in 100 ml. of 1 per cent ethanol. Dissolve 3.63 grams of monopotassium phosphate and 14.35 grams of disodium phosphate in water and dilute to 1 liter. Dilute 4 ml. of bromothymol blue solution to 100 ml. with the phosphate buffer solution. This can be preserved with a drop of chloroform if kept in the refrigerator.

To 20 ml. of clear benzene extract of the base add 2 ml. of bromothymol blue reagent, or sufficient to give a known excess as shown by the color remaining in the aqueous layer. After addition of the reagent to the sample, shake mechanically for 15 minutes and centrifuge. Separate the benzene layer with care. This contains the reacted Paludrin. However, the method is simplified by measuring the color of the aqueous solution of dye. To this aqueous solution add a drop of 40 per cent sodium hydroxide solution and read at 600 mμ against a blank.

As the copper complex. Shake the benzene solution of sample with

1 ml. of a 0.1 per cent aqueous solution of sodium diethyldithiocarbamate. Read the yellow color of the benzene layer against a blank.

N-Methyl-4-phenyl-4-carbethoxypiperidine Hydrochloride, Meperidine Hydrochloride, Demerol

The benzene solubility of the double compound formed by Demerol with bromothymol blue at pH 7.5 is used for its estimation.[112] Various alkaloids other than morphine interfere.

Sample—*Urine.* Adjust 20 ml. to pH 7.5 and extract with 10 ml. and 10 ml. of benzene. Shake the benzene extracts with 10 ml. of 0.05 per cent solution of bromothymol blue. An amount of dye equivalent to the Demerol passes into the benzene to form a yellow compound. Extract the yellow benzene solution with 10 ml. of 0.4 per cent sodium hydroxide solution and read the aqueous layer at 610 mμ.

1,3-Diaza-2,4-cyclopentadiene, Imidazole, Glyoxaline, 1,3-Diazole, Iminazole

There are many derivatives of imidazole. Histidine is the most constant and abundant. Therefore, imidazole derivatives are separated for estimation by diazotized sulfanilic acid.[113] The reaction is applied to the group including their degradation products and the result expressed in terms of histamine hydrochloride. The reaction is the usual one with the color developed by sodium carbonate. The resulting color is unstable and must be read promptly. The minimum amount of imidazoles that can be estimated is 0.006 mg. per ml. .

Sample—*Urine.* Mix 5 ml. of urine with 4 ml. of 40 per cent neutral lead acetate solution. Add 4 ml. of 8 per cent sodium hydroxide solution and centrifuge. Decant through a filter and wash the precipitate twice with water. Precipitate lead in the combined filtrates with a few drops of 20 per cent disodium phosphate solution and centrifuge. Evaporate the decantate and washings at 110°. Take up the residue in water, dilute to 10 ml., and develop with diazotized sulfanilic acid.

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[112] Fred W. Oberst, *J. Pharmacol.* **79**, 10-15 (1943); Robert A. Lehman and Theis Aitken, *J. Lab. Clin. Med.* **28**, 787-93 (1943).

[113] G. Hunter and T. M. Tagarosky, *Can. J. Research* **19B**, 310-17 (1941).

Blood. Use the filtrate from clarification by sodium tungstate and sulfuric acid.

Feces. Disperse in water and treat as for urine.

Procedure—Follow the procedure and reagent for estimation of histamine by *p*-phenyldiazonium sulfonate (page 47) and report in terms of histamine hydrochloride.

2-Benzylimidazoline Hydrochloride

Nitration is used for estimation of benzylimidazoline.[114]

Procedure—Make a sample containing 0.025-0.25 mg. of imidazoline distinctly alkaline by addition of 10 per cent sodium hydroxide. Extract with 10, 10, and 5 ml. of ether. Combine the extracts and evaporate to dryness. Add 0.3 ml. of fuming nitric acid to the residue. Heat on a steam bath until excess acid is evaporated. Take up the residue in 99:1 acetone-absolute ethanol and dilute to 10 ml. with the same solvent. Add 5 drops of 3 per cent potassium methylate in absolute methanol. Mix and read at once at 530 mμ against the solvent mixture.

2-(1-Naphthylmethyl)imidazoline

Extract and develop as described for 2-benzylimidazoline. The color is stable for only 3 minutes.

Indoxyl

Indoxyl reacts with thymol and ferric chloride to give a color extractable with chloroform and suitable for colorimetric estimation.[115] Hemoglobin must be absent in the sample used. Isatin condenses with indoxyl.[116] The condensation reaction requires no oxidation, is a direct measure of one mole of indoxyl equals 1 mole of indirubin, and indirubin is readily available as a standard.

Sample—*Serum.* Mix 5 ml. of serum with 5 ml. of 20 per cent trichloroacetic acid. Filter and use as sample for development with ferric chloride.

Urine. Use undiluted for development by isatin.

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114 Teodor Canbäck, *Farm. Rev.* **45**, 377-80 (1946).

115 Guy Laroche and A. Grigaut, *Diagnostica tec. lab.* (*Napoli*) *Riv. mensile* **4**, 136-9 (1933).

116 James Anthony Drum, *Sci. Proc. Roy. Dublin Soc.* **25**, 295-8 (1951).

Procedure—*By ferric chloride.* To 5 ml. of sample add 1 ml. of a 5 per cent solution of thymol in ethanol. Mix and add 10 ml. of 0.15 per cent ferric chloride solution in concentrated hydrochloric acid. Mix again, let stand for one-half hour, and add 4 ml. of chloroform. Shake, let stand for 5 minutes, and read the chloroform layer against a reagent blank.

By isatin. To 2.5 ml. of sample add 2 ml. of saturated aqueous solution of isatin and 1 ml. of 1:1 sulfuric acid. Heat to boiling over a flame and then heat on a steam bath for 5 minutes. Cool and extract with 5 ml. and 5 ml. of chloroform. Wash the combined chloroform extracts with 10 ml. of 0.1 per cent sodium hydroxide solution. The isatin as isatic acid dissolves in the aqueous layer. Centrifuge the chloroform layer, dilute to 10 ml., and read the indirubin against a reagent blank.

Indole, Benzopyrrole

Indole is a cyclic secondary amine, which is a natural product of pancreatic digestion, bacterial action, and putrefactive decomposition. A major method of determination, after appropriate separation, is by the red color with *p*-dimethylaminobenzaldehyde.[117] Skatole gives the same reaction but is not likely to be present. Urobilinogen also gives the color. The indole is usually separated from interfering substances in the sample by steam distillation. A solution of indole up to 12 per cent of acid leaves the color in the chloroform layer, with much stronger acid as provided in a procedure which follows the color is transferred to the aqueous phase.[118]

One volume of indole solution gives a red with two volumes of methanol and two volumes of fuming hydrochloric acid which is determined at 495 mμ.[119] Colors with such indole derivatives as indoleacetic acid and indolepropionic acid interfere. Tryptophan does not react.

Indole gives a characteristic rose-red on diazotizing to nitroso-

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[117] E. Rohdi, *Z. physiol. Chem.* **44**, 161 (1905); W. E. Marshall, *J. Hygiene* **7**, 581 (1907); Carl R. Fellers and Ray W. Clough, *J. Bact.* **10**, 105-33 (1925); Frank C. Happold and Leslie Hoyle, *Biochem. J.* **28**, 1171-3 (1934); C. B. Allsox, *Ibid.* **35**, 965-6 (1941); R. E. Duggan, *J. Assoc. Official Agr. Chem.* **31**, 507-10 (1948); Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, 7th Ed., pp. 302-4, Association of Official Agricultural Chemists, Washington, D. C. (1950).

[118] L. H. Chernoff, *Ind. Eng. Chem., Anal. Ed.* **12**, 273-4 (1940).

[119] S. Grisolia, *Trabajos inst. nacl. cienc. méd.* (*Madrid*) **3**, 367-72 (1943-44).

indole[120] and with β-naphthoquinone sodium monosulfonate.[121] Indole, skatole, and tryptophan react with xanthydrol in acetic acid[122] and, when heated in the presence of stronger acid such as trichloroacetic[123] or hydrochloric,[124] give a reddish violet. Semicarbizides, hydrazine, and antipyrine interfere. The color of indole with ferric chloride in strong hydrochloric acid solution is extractable with chloroform for reading at 530 mμ.[125]

Samples—*Oyster meat.* Transfer 50 grams to a blender and homogenize with 80 ml. of water. Transfer quantitatively to a flask for steam distillation. Apply sufficient heat to maintain the volume at 80-90 ml. and steam-distil to collect 350 ml. of distillate in about 45 minutes.

Add 5 ml. of 1:20 hydrochloric acid and 5 ml. of saturated sodium sulfate solution. Extract successively with 25, 20, and 15 ml. of chloroform, shaking vigorously at least 1 minute with each. Combine the first two extracts and wash with 400 ml. of water, 5 ml. of 1:20 hydrochloric acid, and 5 ml. of saturated sodium sulfate solution. Wash the 15 ml. extract with the same wash solution. Filter the chloroform extracts and develop with *p*-dimethylaminobenzaldehyde.

Fresh oysters and clams contain no indole. Therefore, the indole content of canned oysters detects the use of decomposed raw oysters.[126] Minor amounts of substances reacting with *p*-dimethylaminobenzaldehyde are present. The same holds for shrimp.[127] There are indications that this is also true of crab meat.

Crab meat. Treat 25 grams as described for oyster meat.

Shrimp. Treat 25 grams as described for oyster meat but with 80 ml. of ethanol. In that case collect 450 ml. of distillate in place of 350 ml.

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[120] Jesse A. Sanders and Clarence E. May, *Biochem. Bull.* **2**, 373-8 (1913).

[121] C. A. Herter and M. Louise Foster, *J. Biol. Chem.* **1**, 257-61 (1905-6).

[122] William Robert Fearon and James Anthony Drum, *Sci. Proc. Roy. Dublin Soc.* **25**, 295-8 (1951).

[123] Mario Zappacosta, *Diagnostica tec. lab.* (*Napoli*) *Riv. mensile* **6**, 870-5 (1935).

[124] Victor Arreguine, *Rev. univ. nacl. Córdoba* (*Arg.*) **31**, 1710-14 (1944); *Rev. asoc. bioquim. argentina* **12**, 3-6 (1945).

[125] Maria Gronwall, *Skand. Arch. Physiol.* **78**, 139-44 (1938).

[126] W. H. King, F. F. Flynn, and James N. Gowanloch, *J. Assoc. Official Agr. Chem.* **28**, 385-98 (1945); L. M. Beacham, *Ibid.*, **29**, 89-99 (1946).

[127] R. E. Duggan and L. W. Strasburger, *Ibid.* **29**, 177-88 (1946).

Bacterial cultures. Acidify 10 ml. of culture with 1 drop of concentrated hydrochloric acid. Extract with two 10-ml. portions of chloroform. Combine the extracts, wash with 10 ml. of water, and develop with *p*-dimethylaminobenzaldehyde.

Plasma. Shake 1 ml. of oxalated plasma in a centrifuge tube with 4.5 ml. of chloroform and centrifuge. Use the clear supernatant liquid for development with *p*-dimethylaminobenzaldehyde.

Blood. Extract 1 ml. of oxalated blood with 3 ml. of chloroform. Repeat the extraction with 2, 2, and 1-ml. portions. Develop the combined extracts with *p*-dimethylaminobenzaldehyde.

Procedure—*By p-dimethylaminobenzaldehyde.* The sample should have been extracted into chloroform. To an appropriate volume of this sample solution containing 0.001-0.01 mg. of indole add 10 ml. of a reagent containing 0.4 gram of *p*-dimethylaminobenzaldehyde in 5 ml. of glacial acetic acid, 92 ml. of 85 per cent phosphoric acid, and 3 ml. of concentrated hydrochloric acid. Shake for exactly 2 minutes and dilute 9 ml. of the acid layer to 50 ml. with glacial acetic acid. Read at 560 mμ, subtracting a reagent blank.

A method for determination with the same reagent in the presence of skatole is given under the latter topic.

By xanthydrol. Mix 2 ml. of aqueous solution containing not over 0.4 mg. of indole with 10 drops of fresh 5 per cent xanthydrol in absolute ethanol. Dilute to 8 ml. with glacial acetic acid free of glyoxylic acid. Heat in boiling water for 10 minutes to develop the color. Cool and read against a reagent blank.

β-Methylindole, Skatole

The reaction of *p*-dimethylaminobenzaldehyde with skatole in the presence of phosphoric acid [128] when dissolved in glacial acetic acid is suitable for colorimetric estimation. The color is stable for hours. By suitable modification the reaction is applicable to indole.

Procedure—*Skatole and indole.* As reagent dissolve 4 grams of pure *p*-dimethylaminobenzaldehyde in 5 ml. of glacial acetic acid on a steam bath and add 100 ml. of 85 per cent phosphoric acid.

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[128] L. H. Chernoff, *Ind. Eng. Chem., Anal. Ed.* 12, 273-4 (1940); Charles S. Myers, *J. Assoc. Official Agr. Chem.* 33, 971-6 (1950).

Mix 50 ml. of sample in chloroform containing 0.03-0.3 mg. of indole with 5 ml. of reagent and shake vigorously for 1 minute. Let it separate or centrifuge, and remove the chloroform layer as completely as possible. Dilute the acid layer to 50 ml. with 85 per cent phosphoric acid and mix. Read after 5 minutes at 565 mμ against a reagent blank.

Indole. To the reagent as described for skatole add 25 ml. of water. Mix 50 ml. of sample in chloroform containing 0.01-0.1 mg. of indole with 5 ml. of reagent. Complete as for skatole starting at ". . . shake vigorously for 1 minute."

Skatole. Subtract the value for indole from that for skatole and indole.

4-Dimethylamino-1,5-dimethyl-2-phenyl-3-pyrazolone, Aminopyrine, Dimethylaminoantipyrine, Pyramidone

Aminopyrine gives a violet color with ferric chloride [129] or a yellow with diazotized *p*-nitroaniline.[130] As another reaction, the familiar phosphotungstic-phosphomolybdic acid reagent is reduced by pyramidone.[131]

Procedure—*By ferric chloride.* Dilute a sample containing 0.1-3 mg. of aminopyrine to about 6 ml. and add 3 ml. of 1 per cent ferric chloride solution. Dilute to 10 ml. and read at once at 570 mμ against a reagent blank.

By diazotized p-*nitroaniline.* Follow the technic for plasmochin (page 310).

By phosphotungstic-phosphomolybdic acid. To a 10-ml. sample containing 5-15 mg. of aminopyrine, add 2 ml. of saturated sodium carbonate solution and 2 ml. of reagent (Vol. III, page 116). Dilute to 15 ml., mix, and read after 20 minutes at 600 mμ against a reagent blank.

1-Phenyl-2,3-dimethylpyrazolone-5-one, Antipyrine

A salmon color with *p*-dimethylaminobenzaldehyde is applicable to estimation of antipyrine.[132] Alternatively biological samples are extracted with chloroform or deproteinized with zinc hydroxide. In either

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129 Fernand Geguin, *Rev. can. biol.* **6**, 36-42 (1947).

130 R. Pulver, *Arch. intern. pharmacodynamie* **81**, 47-52 (1950).

131 T. E. Gulzaeva, *Zhur. Anal. Khim.* **5**, 163-5 (1950).

132 Maurice Pesez, *Ann. chim. anal. chim. appl.* **24**, 11-12 (1942); Guy Deysson, *Ann. pharm. franc.* **7**, 237-42 (1949).

case addition of sodium nitrite to an acid solution forms 4-nitrosoantipyrine, read at 350 mμ against a reagent blank.[133]

Sample—*Biological solutions and dispersions.* Mix 1-5 ml. of sample with 0.5 ml. of 4 per cent sodium hydroxide solution and 20 ml. of chloroform. Shake mechanically for 10 minutes and centrifuge. Evaporate 15 ml. of the solvent layer in a current of air. Take up the residue in 4 ml. of 1:500 sulfuric acid for development with nitrite.

Biological liquids. Mix 2 ml. of sample, 2 ml. of water, and 2 ml. of a 10 per cent solution of zinc sulfate heptahydrate in 1:125 sulfuric acid. Add 2 ml. of 3 per cent sodium hydroxide solution dropwise with mixing and shake for a half-minute. After 10 minutes, centrifuge. To 3 ml. of clear supernatant layer add 1 drop of 1:9 sulfuric acid for development with nitrite.

Procedure—*By p-dimethylaminobenzaldehyde.* Dilute a 1-ml. sample of solution to 10 ml. with water. Add 0.7 ml. of fresh 1.25 per cent solution of *p*-dimethylaminobenzaldehyde in ethanol. Store at 20° for 24 hours or heat at 80° for 1 hour and read at 500 mμ against a reagent blank.

By nitrite. The solution is in dilute aqueous acid. First use as a blank and read at 350 mμ against the same aqueous acid. Add 2 drops of 0.2 per cent sodium nitrite solution to the sample and read again after 20 minutes. Subtract the reading before adding the nitrite.

Pyrimidines

A specific reaction of 2-thiobarbituric acid with the pyrimidine cycle to give a red color is suitable for estimation of various derivatives.[134] If there are substituents in the 4-, 5-, or 6-position, they must be removed if the test is to be applicable. The rate of color development is greatly affected by a functional group, but the molar extinction coefficient when fully developed is identical.

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[133] Julius Axelrod, Robert Soberman, and Betty B. Levy, *J. Biol. Chem.* **179**, 27-9 (1949); Robert Soberman, Bernard B. Brodie, Betty B. Levy, Julius Axelrod, Vincent Hollander, and J. Murray Steele, *Ibid.*, **179**, 31-42 (1949); Cf. M. Delaville, G. Delaville, A. Galli, Hiocco and A. Lichtwitz, *Ann. biol. clin.* (Paris), **10**, 391-6 (1952).

[134] Henry I. Kohn, *Proc. Soc. Exptl. Biol. Med.* **59**, 21-2 (1945); Robert G. Shepherd, *Anal. Chem.* **20**, 1150-3 (1948).

No color or only a yellow is given by 2-amino derivatives of imidazole, 1,3,4-triazine and 1,3,5-triazine, 2-sulfanilamido derivatives of pyrazine, thiazole, 1,3,4-thiadiazole, quinoline, 1-sulfanilamidoquinoline, and sulfanilylguanidine. A transitory pink with 2-sulfanilamidopyridine does not interfere. Other pyridine derivatives do not react.

The nature of the sulfonyl substituents does not affect color formation with 2-phenylsulfonamido-, 2-(3-nitrophenylsulfonamido)-, 2-(4-nitrophenylsulfonamido)-, 2-metanilamido-, 2-sulfanilamido-, 2-(N^3-acetylmetanilamido)-, 2-(N^4-acetylmetanilamido)-, 2-naphthionamido-, 2-taurylamido-, and 2-pantoyltaurylamidopyrimidine. All give the same color characteristics as 2-sulfanilamidopyrimidine and develop completely at 100° in about 50 minutes. It requires 3 hours for complete color development with 2-aminopyrimidine. Acyl, alkyl, and aryl substituents on the amino group do not alter the nature of the reaction.

Procedure—As reagent dissolve 5 grams of highly purified thiobarbituric acid in 5 ml. of 16 per cent sodium hydroxide solution and dilute to 500 ml. Dissolve 37 grams of sodium citrate dihydrate in water, add 32 ml. of concentrated hydrochloric acid, and dilute to 250 ml. Add this to the reagent and adjust the pH to 2.

Adjust the sample to 0.0002-0.002 mg. per ml. Mix 1 ml. with 4 ml. of reagent and heat in boiling water without evaporation, until the maximum color is developed which depends on the compound. Read at 520 mμ. If the color is too faint, extract with 15 per cent of the volume of butanol, extract the color from the separated butanol layer with half its volume of 0.8 per cent sodium hydroxide solution, and mix this alkaline extract with an equal volume of the citrate buffer described earlier.

2,6-Dioxypyrimidine, 2,4(1,3)-Pyrimidinedione, Uracil

Barbiturates react with a cobalt salt in an alkaline medium to give a blue color.[135] A similar color is obtained with —CONHCO— and

[135] James M. Dille and Theodore Koppanyi, *J. Am. Pharm. Assoc.* **23**, 1079-84 (1934); Charles R. Linegar, James M. Dille, and Theodore Koppanyi, *Ibid.* **24**, 847-52 (1935); Melvin W. Green, Fletcher P. Veitch, and Theodore Koppanyi, *Ibid.* **32**, 309-11 (1943); Eugene L. Cohen, *Am. J. Pharm.* **118**, 40-62 (1946); R. W. Merley, *Am. J. Clin. Path.* **18**, 906-9 (1948); Leland N. Mattson and Wendell L. Holt, *J. Am. Pharm. Assoc.* **38**, 55-7 (1949); Wendell L. Holt and Leland N. Mattson, *Anal. Chem.* **21**, 1389-91 (1949).

—CONHCS— groups. Therefore, uracil, thiouracil, and propylthiouracil are determinable to ±2 per cent by the same reaction. The color forms at once and is stable for several hours. The medium is 3:2 anhydrous chloroform-methanol. Moisture causes a gradual fading of the color. Phenobarbital, barbital, pentobarbital, theobromine, phthalimide, alloxan, biuret, and theophylline interfere.

When a neutral sample of uracil is oxidized with bromine, after removal of excess bromine the dibromohydroxyhydro derivative reduces lithium arsenotungstate.[136] Inorganic phosphate interferes,[137] probably by forming phosphotungstate. Cytosine, isocytosine, thiouracil, 5-bromouracil, 5,5-dibromo-6-hydroxyhydrouracil, 5-5-dichloro-6-hydroxyhydrouracil, and 5-bromocytosine interfere after bromination. Isodilauric acid, dilauric acid, barbituric acid, and alloxan do not reduce the reagent before or after bromination. 5-Nitrouracil, isobarbituric acid, cysteine, reduced glutathione, and uric acid reduce the reagent before bromination but not after. Guanine interferes, but adenine does not.

Sample—*Cystosine present.* Shake a 5-ml. sample with 2 grams of Decalso for 5 minutes and centrifuge. The cytosine has been sorbed. Develop a portion of the supernatant liquid by arsenotungstate. This is the uracil. Develop a portion of the original by the same method. The reading is both uracil and cytosine.

Procedure—*By cobalt salt.* Dissolve a dry sample containing about 18 mg. of uracil in dry chloroform and dilute to 25 ml. Mix a 5-ml. aliquot with 5 ml. of a 0.125 per cent solution of anhydrous cobalt acetate in absolute methanol, 5 ml. of 25 per cent isopropylamine in absolute methanol, and chloroform to dilute to 25 ml. Mix and read at 560 mμ. Subtract a reagent blank.

By arsenotungstate. Dilute a sample containing 0.005-0.05 mg. of uracil to 2 ml. and add 7 drops of bromine water. After exactly 5 minutes pass a stream of air through until excess bromine is gone. Add 3 ml. of water and 5 ml. of 2.5 per cent sodium cyanide solution containing 25 per cent of urea. Add 1.5 ml. of lithium arsenotungstate reagent (Vol III, page 438). After 1 hour for color development dilute to 25 ml. and read at 660-690 mμ against a reagent blank.

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[136] Morris Soodak, Anthony Pircio, and Leopold Cerecedo, *J. Biol. Chem.* **181**, 713-18 (1949).

[137] Arthur K. Saz, *Arch. Biochem. Biophys.* **35**, 469-70 (1952).

2,4-Dihydroxy-5-methylpyrimidine, 5-Methyluracil, Thymine

Thymine is the characteristic pyrimidine of desoxyribosenucleic acid. When thymine is coupled with diazotized sulfanilic acid in sodium carbonate solution in air and sodium hydroxide and hydroxylamine added, it develops a red color.[138] Coupling requires several hours, but this can be cut to 20 minutes under oxygen at 30°.[139] Some coupling also occurs with cytosine and uracil. The instability of color is corrected by use of glycerol and sodium hydroxide.

Extraction of vegetable nucleic acids from tissue with trichloroacetic acid alters their structure[140] as indicated by the Feulen reaction[141] with reduced fuchsin.

Sample—*Nucleic acid.* Seal a weighed sample in a tube with 4 ml. of 1:3 sulfuric acid and heat at 125-130° for 24 ± 2 hours. Cool, open, and transfer with 16 ml. of water in 1-ml. portions. Add excess silver sulfate to precipitate silver purines. Refrigerate overnight, centrifuge, and decant. Wash the precipitate twice with 1:19 sulfuric acid saturated with silver sulfate. Combine the decantate and washings and precipitate silver with hydrogen sulfide. Centrifuge, decant, and boil off hydrogen sulfide. Cool and neutralize with sodium carbonate.

Extract aldehydic derivatives of desoxyribose with ether by two extractions. Dilute the aqueous layer to a known volume for the use of aliquots.

Procedure—As reagent mix 6 ml. of 0.9 per cent sulfanilic acid monohydrate solution in 9:91 hydrochloric acid with 6 ml. of 0.5 per cent sodium nitrite solution. After 10 minutes add 24 ml. more of the nitrite solution and dilute to 100 ml. with water. Store in an ice bath and use 1-48 hours after preparation.

Fill a Klett tube with water and seal with a serum stopper. Introduce oxygen through one hypodermic needle and let the displaced water pass out through another. Add 2 ml. of sample mixed with 4 ml. of 1.2 per cent sodium carbonate solution. Add 2 ml. of the diazo reagent. Store at 30° for 20 ± 2 minutes. A deep yellow develops. Add 2 ml. of

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138 George Hunter, *Biochem, J.* **30**, 745-7 (1936); D. L. Woodhouse, *Ibid.* **44**, 185-7 (1949).

139 Eugene D. Day and William A. Mosher, *J. Biol. Chem.* **197**, 227-32 (1952).

140 Emile Michel-Durand, *Compt. rend.* **204**, 613-15 (1937).

141 Cf. Hans Ris and A. E. Mirsky, *J. Gen. Physiol.* **33**, 125-46 (1949).

fresh 1:1 mixture of 24 per cent sodium hydroxide and glycerol, and mix. The red color develops within 1 hour. Read at 520 mμ against a reagent blank.

4-Amino-2(1)-pyrimidone, 4-Amino-2-oxo-1,2-dihydropyrimidine, Cytosine

This compound is determined by arsenotungstate according to the technic for uracil. Each is determined by difference before and after sorbing out the cytosine.

6,7-Dimethyl-9-(D-1′-ribityl)isoalloxazine, Riboflavin, Vitamin B_2

Riboflavin can be read directly,[142] but the method has been superseded by the yellow-green fluorescence at 430-440 mμ,[143] the intensity being affected by the solvent.[144] The maximum in aqueous solution is at pH 6-7. The solution conforms to Beer's law over the range 0.000013-0.00013 mg. per ml.[145] The fluorescence is decreased by halides, cyanides, thiocyanates, nitrites, sulfites, ferrous ion, and ferric ion.[146] It is not readily destroyed by oxidation or reduction. Reduced to a nonfluorescing form with very strong agents such as sodium hydrosulfite, it is reoxidized by shaking with air. It is not reduced by stannous chloride.[147] Rapid determination is required as the wave lengths which cause fluorescence also cause decomposition. Visual evaluation com-

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[142] Abram Yu. Kharit and Nicolay V. Khaustov, *Biochem. J.* 29, 34-7 (1935); W. Koschara, *Z. physiol. Chem.* 232, 101-16 (1935).

[143] R. Kuhn and T. Wagner-Jauregg, *Ber.* 67B, 361-3 (1934), W. Koschara, *Z. physiol. Chem.* 232, 101-16 (1935); A. Emmerie, *Nature*, 138, 164 (1936); *Acta Brevia Neerland Physiol., Pharmacol., Microbiol.*, 6, 108-9 (1936); Hans von Euler and Erich Adler, *Ark. Klini, Mineral· o. Geol.* 11B, No. 28 (1934); *Z. physiol. Chem.* 223, 105-12 (1934); F. H. Cohen, *Acta Brevia Neerland. Physiol., Pharmacol., Microbiol.*, 4, 46 (1934); *Rec. trav. chim.* 54, 133-8 (1935); Francisco Vivanco, *Naturwissenschaften*, 23, 306 (1935); G. C. Supplee, S. Ansbacher, G. E. Flanigan, and Z. M. Hanford, *Dairy Sci.* 19, 215-20 (1936); G. Narasimhamwethy, *Indian J. Med. Research* 24, 1083-92 (1937); Joseph W. Ferrebee, *J. Clin. Inv.* 19, 251-6 (1940); Michinori Takada, *J. Japan. Soc. Food Nutrition* 2, 181-2 (1950).

[144] Paul Karrer and H. Fritzsche, *Helv. Chim. Acta* 18, 911-14 (1935); Richard Kuhn and Giovanni Moruzzi, *Ber.* 67B, 888-91 (1934).

[145] R. T. Conner and G. J. Straub, *Ind. Eng. Chem., Anal. Ed.* 13, 385-8 (1941).

[146] P. Ellinger and M. Holden, *Biochem. J.* 38, 147-50 (1944).

[147] A. Z. Hodson and L. C. Norris, *J. Biol. Chem.* 131, 621-30 (1939).

petes with photometric estimation but is less reliable. Aside from natural standards uranium glass and solutions of potassium dichromate and fluorescein are used.[148]

A permanganate treatment of the riboflavin solution, followed by reduction of the excess, destroys some interfering pigments without destruction of riboflavin, if properly conducted.[149] Some deeply colored samples such as meat extracts require the treatment. Reduction followed by air oxidation, if repeated before the fluorescence is read, reduces the blank. When sorption followed by elution is practiced, the most common sorbant is Florasil and the eluting agent pyridine in acetic acid. High results due to sorption of foreign materials sometimes occur. Sorption on synthetic cation-exchange resins derived from *o*- and *p*-phenolsulfonic acids[150] or on prepared Frankonite[151] is also successful.

As a general principle, substances with stronger sorption than riboflavin are removed on pyridine-treated zeolite; those equal to or less than that of riboflavin come out on the synthetic resin. Those weaker than riboflavin are then removed by rinsing with hot water. The remainder is eluted with pyridine-acetic acid. Those not resistant to oxidation are destroyed by potassium permanganate. Finally, fluorescence other than that around 560 mμ is screened out.

Pyridine treatment reduces the affinity of zeolite for riboflavin. Any riboflavin sorbed is easily eluted with water without loss of thiamine. Recovery of riboflavin is over 90 per cent. The zeolite chromograph of thiamine[152] can be used for its determination on the same sample. Amberlite IR-100 also sorbs riboflavin.[153]

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148 F. H. Cohen, *Rec. trav. chim.* **54**, 133-8 (1935); S. M. Weissberg and I. Levin, *Ind. Eng. Chem., Anal. Ed.* **9**, 523-4 (1937); D. B. Hand, *Ibid.* **11**, 306-9 (1939); J. S. Andrews, *Cereal Chem.* **20**, 613-25 (1943).

149 Walter Koschara, *Ber.* **67B**, 761-6 (1934); Royal A. Sullivan and L. C. Norris, *Ind. Eng. Chem., Anal. Ed.* **11**, 535-40 (1939); Sdun'ichi Kozuka and Yoshitsuga Nose, *Igaku to Seibutsugaku* (Med. and Biol.) **16**, 56-7 (1950); Kenji Matsuura, *Ibid.* **16**, 63-5 (1950).

150 Friedrich Weygand and Karl Wacker, *Klin. Wochschr.* **24/25** 438-40 (1947); W. Neuweiler and W. Ritter, *Intern. Z. Vitaminforsch.* **22**, 387-92 (1951).

151 Montonori Fujiwara and Hiroshi Shimizu, *Anal. Chem.* **21**, 1009-11 (1949).

152 Douglas J. Hennessy, *Ind. Eng. Chem., Anal. Ed.* **13**, 216-8 (1941); Motonori Fujiwara and S. Kitamura, *Japan. J. Hyg.* **2**, 12 (1948).

153 Donald S. Herr, *Ind. Eng. Chem.* **37**, 631-4 (1945); Elmer B. Brown, Albert F. Bina, and James M. Thomas, *J. Biol. Chem.* **158**, 455-61 (1945).

Enzymatic liquefaction of large amounts of starch is helpful.[154] Fluorescence is read visually or photometrically. A non-recoverable ester fraction is broken down to riboflavin by enzyme action.[155]

In milk, riboflavin is sometimes referred to as lactoflavin.[156] Fluorometric methods are preferred for dried milk[157] and agree with microbiological results in the absence of ether-soluble materials.[158] Strong acidification of the sample after reading suppresses the fluorescence due to riboflavin to give another method of getting a blank reading.[159]

Riboflavin can be separated from an aqueous medium by addition of pyridine and saturation of the mixture with anhydrous sodium sulfate. Appropriate quantities of this salt causes pyridine and water to become immiscible and the pyridine forms a separate layer containing most of the riboflavin.[160] Isobutanol separates the rest of the riboflavin from the aqueous phase. The riboflavin is then measured in the pyridine-isobutanol solution. Results are in good agreement with the best microbiological methods.[161]

Irradiation of riboflavin in alkaline solution splits off a side chain to give lumiflavin. This is then extracted with chloroform from an acidified solution for estimation.[162] The conversion is not quantitative. Therefore, results are comparative against a standard similarly treated.

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[154] John S. Andrews, Harold M. Boyd, and David E. Terry, *Ind. Eng. Chem., Anal. Ed.* **14**, 271-4 (1942).

[155] Lawrence Rosner, Evelyn Lerner, and Howard J. Cannon, *Ibid.* **17**, 778-80 (1945).

[156] David B. Hand, *Ibid.* **11**, 306-9 (1939).

[157] A. K. Kemmerer, *J. Assoc. Official Agr. Chem.* **23**, 246-51 (1940).

[158] Royal A. Sullivan, Annabel Beaty, Evelyn Bloom, and Earl Reeves, *Arch. Biochem.* **2**, 333-43 (1943).

[159] A. Gourévitch, *Bull. soc. chim. biol.* **30**, 711-12 (1948)

[160] Victor A. Najjar, *J. Biol. Chem.* **141**, 355-64 (1941); Robert E. Johnson, Frederick Sargent, Paul F. Robinson and Frank C. Consolazio, *Ind. Eng. Chem., Anal. Ed.* **17**, 384-86 (1945); E. C. Slater and D. B. Morell, *Biochem. J.* **40**, 644-52 (1946).

[161] Elmer De Ritter, Mary E. Moore, Erich Hirschberg, and Saul H. Rubin, *J. Biol. Chem.* **175**, 883-92 (1948).

[162] Hans von Euler, Erich Adler, and Arnulf Schlötzer, *Z. physiol. Chem.* **226**, 87-94 (1934); Richard Kuhn, Theodor Wagner-Jauregg and Hans Kaltschmitt, *Ber.* **67B**, 1452-7 (1934); Francisco Vivanco, *Naturwissenshaften* **23**, 306 (1935); A. J. Charite and N. W. Khaustov, *Compt. rend. acad. sci.* (USSR) **1**, 177-80 (19365; H. Roth, *Vorratspflege u. Lebensmittelforsch.* **4**, 34-43 (1943); Y. L. Wang, K. S. Ting, H. Lih, J. Chao, and Y. H. Hu, *Science and Technol.* (China) **1**, 33-4 (1948).

Preliminary extraction of fluorescent substances with chloroform is necessary for urine and feces and desirable with many other biological samples.[163] Chloroform extraction in the alkaline medium after photolysis does not remove the blank-producing substances. The lumiflavin produced is extractable from acid solution with chloroform. The photolysis is preferably at 20-40° in 2 per cent sodium hydroxide solution at 20 cm. distance with 200 watts for 30-120 minutes. A brick-red with 10 per cent silver nitrate in neutral solution is read at 530 mμ.[164]

Sample—*Bread or whole wheat flour.* Autoclave a 5-gram sample with 50 ml. of 1:360 sulfuric acid for 15 minutes at 15 pounds pressure. When cooled to 50° add 12 ml. of 6.5 per cent trisodium phosphate dodecahydrate solution and check that the pH is approximately 6. Add 1 ml. of fresh 6 per cent takadiastase solution and set aside in the dark for 45 minutes at room temperature. Dilute to 100 ml., filter, and discard the first 20 ml.

Sorb the riboflavin by passing a 20-ml. aliquot through a 65-mm. column of Florasil in a tube 6 mm. in diameter with a reservoir at the top and a capillary at the bottom to permit passage at 1 ml. per minute. Wash with 10 ml. of hot water and discard the liquids. Dry by drawing air through the column and elute with 15 ml. of a 20 per cent solution of pyridine in 1:50 acetic acid. Dilute to 20 ml. with that solvent and filter if cloudy. Use for direct reading of the fluorescence. With enriched bread permanganate treatment of the filtrate removes some interfering substances.

Flour premixes. Prepare the extracts as described for thiamine (page 89), the effect of iron being avoided by the technics described. Read fluorometrically without reduction.

Dry or semidry samples lacking basic substances.[165] Add 1:120 hydrochloric acid amounting to not less than 10 times the dry weight of the sample. Disperse and wash down with 1:120 hydrochloric acid. Autoclave at 15 pounds for 30 minutes and cool. Disperse, add 4 per cent sodium hydroxide solution to pH 6, and 1:120 hydrochloric acid to pH 4.5. Dilute to a volume which contains no more than 0.1 mg. of riboflavin per liter and filter or centrifuge. Adjust an aliquot of the clear solution to pH 6.8 with 4 per cent sodium hydroxide solution.

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[163] Kunio Yagi, *Igaku to Seibutsugaku*, **18**, 264-6 (1951).

[164] M. Z. Barakat and N. Badran, *J. Pharm. Pharmacol.* 3, 501-5 (1951).

[165] Anon., *J. Assn. Official Agr. Chem.* 32, 108-10 (1949).

Concentrate *in vacuo* and dilute to approximately 1 mg. of riboflavin per liter. Develop the fluorescence after oxidation.

Samples containing basic substances. Neutralize a weighed sample to pH 6. Add water to total 10 times the weight of sample. Add 1 ml. of concentrated hydrochloric acid per ml. of solution and proceed as for samples lacking basic substances, starting at "Disperse and wash down . . . ."

Green vegetation.[166] Reflux a sample weighing 10-20 grams with 150 ml. of 70 per cent methanol for 45 minutes. Cool, centrifuge, and wash the residue with about 20 ml. of 70 per cent methanol. Evaporate *in vacuo* to about 25 ml. and extract with 20, 10, 10, and 10 ml. of low-boiling petroleum ether. Discard the methanol layer and extract the hydrocarbon solution with 20, 10, 10, and 10 ml. of chloroform until the solution is colorless. Treat with ultraviolet radiation to convert to lumiflavin and read fluorimetrically.

Tea.[167] Mix 100 ml. of water, 0.1 gram of commercial diastase, and 1 gram of tea. Adjust the pH to 4.5 with 1:3 hydrochloric acid and add a few ml. of toluene. Incubate at 37° for 15 hours. Heat to 80° for 15 minutes, cool, and filter. Mix 2 ml. of filtrate with 2 ml. of 4 per cent sodium hydroxide solution and expose to a 200-watt lamp at 20 cm. for 2 hours. Acidify with acetic acid and oxidize with excess of 4 per cent potassium permanganate solution. Remove the excess permanganate with 3 per cent hydrogen peroxide after 2 minutes. Extract with chloroform and read as lumiflavin.

Feedstuffs;[168] *molasses substantially absent.* Weigh out a 5-gram sample and add, with stirring, 50 ml. of 1:125 sulfuric acid, breaking up any lumps which form. Reflux for 1 hour and allow the extract to come to room temperature. Adjust the pH to 7-7.5 with trisodium phosphate solution, using nitrazine paper as an indicator. Add water so that the total liquid added is 100 ml. Filter after 30 minutes. Pipet out an aliquot of the filtrate estimated to contain 0.2 mg. of riboflavin and dilute to about 175 ml. Add 2 ml. of 2.5 per cent sodium hydrosulfite solution containing 2 per cent of sodium bicarbonate and 2 ml. of 50.08 per cent stannous chloride solution in 1:120 hydrochloric acid. Mix and dilute to 200 ml. After 10 minutes, shake vigorously in a liter

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[166] H. Roth, *Biochem, Z.* **320**, 355-8 (1950).

[167] Tsuru Wada and Yoshito Sakurai, *J. Sci. Research Inst.*, (Japan), **46**, 36-42 (1952).

[168] Cf. J. H. Schutinga, *Rev. trav. chim.* **61**, 359-64 (1942).

container with access to air. If a precipitate forms during the reduction and oxidation, filter the solution. Avoid exposure to light during this procedure. Read the fluorescence.

Feedstuffs containing molasses. If the sample contains large amounts of molasses, a modification is made in the preceding treatment at "Filter after 30 minutes." Add 10 ml. of neutralized extract to 90 ml. of methanol. Mix and filter, avoiding evaporation. Continue with "Pipet out an aliquot of the filtrate . . . ." It is not advisable to precipitate with methanol if avoidable because riboflavin is sometimes lost on the precipitate.

Alternatively the extraction of a 5-gram sample may be made by refluxing with 50 ml. of a 1:130 dilution of sulfuric acid with 50 per cent acetone for 30 minutes.[169] After two extractions, evaporate the combined extracts *in vacuo* and take up in 50 ml. of water as the sample.

Samples below 0.005 mg. per gram. Erroneously low results are due to incomplete extraction and the presence of interfering pigments.[170]

Acid extraction. Mix not over 20 grams of sample, preferably containing about 0.035 mg. of riboflavin, with 150 ml. of 1:360 sulfuric acid. If the sample is not very finely pulverized, mix in a blender. Immerse in boiling water with frequent swirling for 45 minutes, reflux for 45 minutes, or autoclave at 15 pounds pressure for 20 minutes, and cool.

If the sample is to be treated with enzyme, skip to the next subtitle. Otherwise add 10 ml. of 34 per cent sodium acetate trihydrate solution. If the sample has coagulated during heating, macerate again in the blender. Dilute with water to 250 ml. and filter.

Enzyme treatment. At the point ". . . for 20 minutes and cool," add 3 per cent of the weight of sample as pancreatic diastase (clarase)[171] dispersed in 10 ml. of 34 per cent sodium acetate trihydrate solution. Redisperse with a blender if necessary and digest at 45-50° for 2 hours. Dilute with water to 250 ml. and filter. The filtrate, either with or without enzyme treatment, can be used directly as sample, but permanganate treatment permits greater accuracy.

Permanganate oxidation. Measure out two 50-ml. aliquots, designated U and R. To R add 0.5 ml. of riboflavin standard containing

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[169] E. Kodicek and Y. L. Wang, *Biochem. J.* **44**, 340-3 (1949).

[170] S. H. Rubin, E. DeRitter, R. L. Schuman, and J. C. Bauernfeind, *Ind. Eng. Chem., Anal. Ed.* **17**, 136-40 (1945).

[171] Walter J. Peterson, D. E. Brady, and A. O. Shaw, *Ibid.* **15**, 634-6 (1943).

0.015 mg. per ml. in 1:700 sulfuric acid. To each flask add 2 ml. of 4 per cent potassium permanaganate solution, more if necessary, and swirl. After 2 minutes titrate out the excess permanganate with 3 per cent hydrogen peroxide. If a precipitate forms, titrate so long as progressive diminution of color and solution of precipitate occurs. Use care to avoid a residual excess of hydrogen peroxide and if necessary back-titrate with a drop or two of the permanganate solution.

Transfer quantitatively to 100-ml. glass-stoppered cylinders, dilute to 20 ml., and add a drop of caprylic alcohol. Filter if necessary, and if filtration is difficult add enough strong trisodium phosphate solution to make the precipiatate flocculate, correcting for it in the rated volume of solution. Lots U and R are now ready for fluorometry. R serves to demonstrate that the recovery after treatment is satisfactory.

Yeast.[172] Gently reflux a 10-gram sample with 100 ml. of 1:20 hydrochloric acid for 40 minutes and cool. Stopper tightly and place in a refrigerator for 1 hour. Filter the supernatant liquid through a fluted filter.

Adjust the pH of a 25-ml. aliquot of the filtrate to 3.5-4 by adding 2.25 ml. of 20 per cent sodium hydroxide solution. Adjust the volume to nearly 49 ml. with water. Add 1 ml. of a solution containing 5 per cent of sodium hydrosulfite and 5 per cent of sodium bicarbonate. This reduces interfering pigments. Dilute the colorless solution to 50 ml. After 10 minutes in a stoppered container, shake vigorously with access to air for 5 minutes. This reoxidizes the riboflavin to the fluorescent form. Interfering pigments are not reoxidized. Filter the solution through a Gooch crucible with an asbestos mat, using moderate suction. Read the fluorescence without further reduction and oxidation.

Bacteria.[173] Mix 2 ml. of an appropriate suspension with 2 ml. of 4 per cent sodium hydroxide solution. Irradiate this as well as a standard and a blank at 20-40° for 30-60 minutes at 20 cm. from a 200-watt lamp. Add 0.2 ml. of glacial acetic acid and cool to 10°. Extract with 4 ml. of chloroform for reading fluorometrically.

Tissue.[174] Heat with an appropriate amount of water at 80° for about 5 minutes and homogenize. Heat the homogenate at 80° for 15

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[172] A. E. Schumacher and G. F. Heuser, *Ibid.* **12**, 203-4 (1940).

[173] Kunio Yagi and Susumu Mitsuhashi, *Japan. J. Exptl. Med.*, **21**, 353-60 (1951).

[174] Kunio Yagi and Isao Ishiguro, *Igako to Seibutsugaku*, **17**, 105-7 (1950).

minutes and centrifuge. Conjugated riboflavin is not hydrolyzed in the process.

Liver.[175] The riboflavin in liver is sometimes called hepatoflavin. Grind 10 grams of liver with 100 ml. of 75 per cent methanol and digest at 37° for 24 hours. Filter and add 2 ml. of glacial acetic acid and 2 ml. of saturated potassium permanganate solution to a 20-ml. aliquot. After 10 minutes remove the permanganate color by titrating with 3 per cent hydrogen peroxide. Read the fluorescence against a reagent blank without prior reduction.

Meat and meat products.[176] Drop a sample weighing 10-20 grams into 100 ml. of 1:900 sulfuric acid in a blender and macerate for 2 minutes at high speed. Transfer the resulting creamy mixture quantitatively with the minimum amount of water. Stopper the container with cotton, autoclave for 15 minutes at 15 pounds, and cool.

Prepare a buffer for pH 4.5 containing 54.4 ml. of glacial acetic acid and 66.94 grams of anhydrous sodium acetate per liter. Add to the sample 20 ml. of a 2.5 per cent solution of pancreatic diastase (Clarase)[177] freshly prepared in the buffer. Mix thoroughly and incubate at 45° for 24 hours. Agitate two or three times during the incubation.

Dilute the extract to 200 ml. Mix well either by shaking manually or by means of a blender for 30 seconds. Filter or clarify by centrifugation for reading the fluorescence, without reduction, against a reagent blank. The enzyme will often contribute 15-25 per cent of the total sample reading.

Eggs.[178] Hard-boil the eggs for 6-10 minutes, peel, and separate the yolks from the whites for separate determination.

White. Treat as described for meat and meat products.

Yolk. Treat as described for meat and meat products until the final extract is prepared at ". . . blender for 30 seconds." This is a stable emulsion which cannot be clarified by centrifugation or filtration. To the entire extract in a blender, add 10 ml. of chloroform and mix for 30 seconds. Transfer to a flask and allow to stand in the dark for at

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175 M. von Eekelen and A. Emmerie, *Acta. Brevia Neerland. Physiol., Pharmacol., Microbiol.* **5**, 77-8 (1935).

176 Walter J. Peterson, D. E. Brady and A. O. Shaw, *Ind. Eng. Chem., Anal. Ed.* **15**, 634-6 (1943); Cf. Winifred F. Hinman, Ruth E. Tucker, Loretta M. Jans, and Evelyn G. Halliday, *Ibid.* **18**, 296-301 (1946).

177 R. T. Conner and G. J. Straub, *Ibid.* **13**, 385-8 (1941).

178 Cf. T. Barton Mann, *Analyst* **21**, 166-71 (1946).

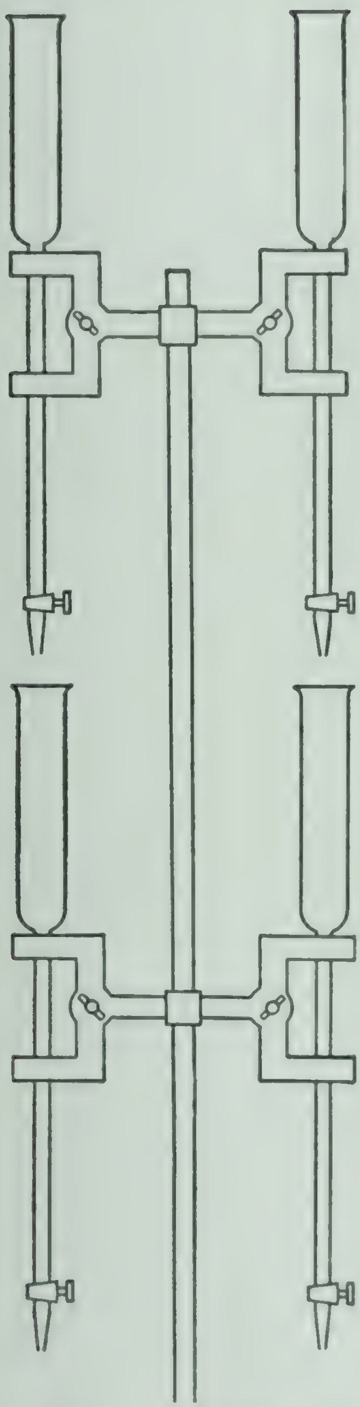

FIG. 14. Exchange tube assemblies

least 30 minutes, then filter. Collect about 20 ml. of filtrate for the assay. This filtrate can be kept under refrigeration for several days without deterioration before reading the fluorescence, without reduction, against a reagent blank.

Alternatively, pipet 5 ml. of well-mixed egg-yolk extract, add 10 ml. of acetone, mix, and filter. Make fluorescence reading on the filtrate immediately, without reduction, against a reagent blank.

Rice, soybeans, vegetables, fruit, nuts. Prepare two exchange assemblies of brown glass, about 8 mm. in diameter, as shown in Figure 14. Fill the upper column of each with 1.5 grams of activated 60-100 mesh zeolite. Fill the lower part of each with 1.5 grams of base-exchange resin. This assembly can be used about 500 times and completely sorbs riboflavin. Treat the zeolite with 50 ml. of 10 per cent pyridine in glacial acetic acid and then wash each with about 200 ml. of water. Treat the resin with hot water, then with 1:1 pyridine-water until no fluorescence results on elution with 1:3 pyridine-glacial acetic acid. Save this eluate as a blank and rinse with about 200 ml. of water.

Transfer a pulverized sample containing 0.002-0.005 mg. of riboflavin with about 40 ml. of water. Add 1:10 hydrochloric acid or 4 per cent sodium hydroxide solution to adjust to pH 4.5. Add 2 ml. of 2 per cent takadiatase solution which has been filtered through both zeolite and resin columns. Add a few drops of toluene and incubate at 38° overnight. Add 5 ml. of 1:35 sulfuric acid and stir in boiling water for 15 minutes. Cool, dilute to 50 ml. and centrifuge. Precipitate proteins from the clear centrifugate with the minimum effective amount of fresh 10 per cent metaphosphoric acid solution.

Adjust the pH to 4-5 with 4 per cent sodium hydroxide solution, centrifuge, and pass a clear aliquot containing 0.001-0.003 mg. of riboflavin through the two columns at 1-2 ml. per minute. Wash successively six times with 5-ml. portions of water at the same rate. Thiamine is sorbed on the zeolite; some other substances less strongly sorbed will be with the riboflavin on the resin. Wash the resin with hot 0.2 per cent acetic acid until the solution coming through does not fluoresce.

Elute the resin column with 25 ml. of 1:4 pyridine-acetic acid at 1 ml. per minute. This contains the riboflavin. Dilute to exactly 25 ml. and mix 4 ml. of eluate with 0.1 ml. of glacial acetic acid. Add 0.15 ml. of 4 per cent potassium permanganate solution prepared fresh weekly. After 8 minutes add 3 per cent hydrogen peroxide, prepared by dilution of 30 per cent hydrogen peroxide, titrametrically until the permanganate is removed.

Urine. Macro. Store the sample in a dark bottle with one-thirtieth its volume of glacial acetic acid as preservative. If riboflavin deficiency is suspected, use a 5-ml. sample, otherwise 1-2 ml. diluted to 5 ml. Add 1 ml. of glacial acetic acid. Next add 2 ml. of purified pyridine and shake well. Add 1-2 drops of 4 per cent potassium permanganate solution for every ml. of urine used. After 1 minute, add 3 per cent hydrogen peroxide to just decolorize the solution. Add 5 grams of anhydrous sodium sulfate and 10 ml. of butanol. Shake vigorously for 2 minutes and centrifuge for 10-15 minutes. Pipet off the butanol as sample, for reading fluorescently without prior reduction.

Alternatively, if riboflavin is less than 0.0001 mg. per ml. or if the sample is highly colored, prepare freshly precipitated lead sulfide by passing hydrogen sulfide through 40 ml. of a 5 per cent solution of lead acetate in 0.25 per cent acetic acid. Wash the lead sulfide 4 times with 300 ml. of water, allow the precipitate to settle, and decant the supernatant liquid. Add 10-30 ml. of urine to the moist precipitate and agitate gently for 10 minutes. Filter with slow suction through a glass filter. Wash the lead sulfide 3 times with 30 ml. of water, leaving just enough water on the surface of the precipitate so that cracks do not form.

Prepare an eluting solution with 70 ml. of water, 30 ml. of pyridine, and 2 ml. of glacial acetic acid. Add 20 ml. of this eluting solution and allow it to pass through the lead sulfide layer and filter drop by drop under slow suction. Add 3 ml. of glacial acetic acid to the eluate and

complete as above, starting at "Next add 2 ml. of purified pyridine . . . ."

Micro.[179] Add 0.1 gram of oxalic acid as stabilizer to each 25 ml. of freshly voided urine. Riboflavin and nicotinamide are stable for days and thiamine for weeks. Store in brown bottles and operate in diffuse light.

To a 0.5-ml. sample add 0.5 ml. of 1:1 pyridine-glacial acetic acid. Add a drop of 4 per cent aqueous potassium permanganate and mix. After 1-5 minutes add 2 drops of 3 per cent hydrogen peroxide and mix. If the color of permanganate is not completely destroyed, add another drop of peroxide. Add 1.5 ml. of isobutanol and shake vigorously. After 1-5 minutes the two phases will have nearly separated and the riboflavin is in the solvent layer. Add a small amount of anhydrous sodium sulfate and agitate gently to clear the solvent layer. In 1-2 minutes it should be clear; if not, centrifuge gently. Use this solvent layer for reading fluorescence by the micro method. It is stable for at least 2 hours.

For development as lumiflavin, add concentrated hydrochloric acid to a 25-ml. sample until the pH is 3-4. Shake with 15 ml. of chloroform for 1 minute and centrifuge to separate. Transfer 10 ml. of the aqueous upper layer to each of two Petri dishes and add 0.002 mg. of riboflavin in aqueous solution to the one to be standard. Add 4 ml. of 20 per cent sodium hydroxide solution to each and irradiate with occasional agitation for 25 minutes. This converts riboflavin to lumiflavin. Again acidify to pH 3-4. Extract each with 5 ml. of chloroform, and repeat. Adjust the combined chloroform layers to 10 ml.

Blood serum. Mix 10 ml. of 15 per cent trichloroacetic acid with 3 ml. of sample to precipitate proteins. Use a 5-ml. aliquot of the clear upper layer for completion by the macro method described for urine, starting at "Add 1 ml. of glacial acetic acid . . . ."

Whole blood. Mix 10 ml. with 30 ml. of methanol and a few crystals of ammonium oxalate. After 30 minutes at 60°, filter and wash the filter until the filtrate totals 60 ml. Add 2 ml. of glacial acetic acid and filter again. Add 1 ml. of 4 per cent potassium permanganate solution to the filtrate and after 2 minutes add 1 ml. of 3 per cent hydrogen peroxide. Filter and concentrate *in vacuo* to 20 ml. Dilute to 120 ml. with water and shake with about 5 grams of Frankonite to sorb the

[179] Robert E. Johnson, Frederick Sargent, Paul F. Robinson, and Frank C. Consolazio, *Ind. Eng. Chem., Anal. Ed.* 17, 384-6 (1945).

riboflavin. Let settle and decant the liquid. Wash the Frankonite with water, separating by centrifuging, until the washings are chloride-free. To elute the riboflavin, treat the precipitate with 10 ml. of 20 per cent aqueous pyridine containing 2 per cent of acetic acid. Centrifuge, decant, and extract with another 10 ml. of the solvent. Filter the combined extracts for reading in pyridine.

Milk. Centrifuge to separate the fat and add 8 ml. of 10 per cent trichloroacetic acid to 2 ml. of the resulting skim milk. Filter off precipitated protein and use 5 ml. of the filtrate by the macro method described for urine, starting at "Add 1 ml. of glacial acetic acid . . . ." If the riboflavin content is low, repeat using 15 ml. of 10 per cent trichloroacetic acid to 10 ml. of milk, let stand for an hour to coagulate, and filter.[180]

Alternatively, mix 50 ml. of acetone with 25 ml. of whole milk and filter, refiltering the first few ml.

Procedure [181]—*In aqueous solution without prior reduction.* Being sure that the solvent shows no fluorescence, read the fluorescence value A after standardizing against pure sodium fluorescein. Add a solution containing 0.0015 mg. of riboflavin to the amount of 0.0001 mg. per ml. of sample solution and read value B. Add 10-20 mg. of solid sodium hydrosulfite, stir until dissolved, and read value C.

$$\text{Riboflavin} = \frac{\text{A} - \text{C (dilution factor)}}{\text{B} - \text{A (dilution factor)}}\ 0.0001 \text{ ml. of aliquot}$$

After reduction. A further reduction followed by oxidation can be applied to the prepared sample. To 50 ml. of sample solution add 1 ml. of fresh 2.5 per cent sodium hydrosulfite solution containing 2 per cent of sodium bicarbonate. Add 2.5 ml. of a stannous chloride solution prepared by 1:500 dilution of a stock of 10 grams in 25 ml. of concentrated hydrochloric acid. After 10 minutes, shake vigorously with exposure to air to reoxidize the riboflavin and read the fluorescence.

In aqueous solution with oxidation. Measure out 4 portions of 10 ml. of sample solution. To each of 2 tubes add 1 ml. of riboflavin solution containing 0.001 mg. of riboflavin per ml. Add 1 ml. of water to

[180] C. H. Whitnah, Bernice L. Kunerth, and M. M. Kramer, *J. Am. Chem. Soc.* **59**, 1153-4 (1937); Cf. Gulbrand Lunde, Hans Kringstad and Alt Olsen, *Z. physiol. Chem.* **260**, 141-7 (1939).

[181] J. S. Andrews, *Cereal Chem.* **20**, 3-23 (1943).

the other tubes. To each tube add 1 ml. of glacial acetic acid, mix, and add 0.5 ml. of 4 per cent potassium permanganate solution. Mix and after 2 minutes add 0.5 ml. of 3 per cent hydrogen peroxide. The color disappears within 10 seconds after mixing. Shake to eliminate oxygen bubbles and read as A, a sample with added riboflavin. Likewise read a sample with added water as B. Add 20 mg. of sodium hydrosulfite to the other 2 tubes, mix and read within 5 seconds as C. Then mg. per ml. in final sample solution = $0.1 \times 0.001 \times (B - C)/(A - B)$.

In isobutanol. Macro. Read the fluorescence of the sample with a mercury-vapor lamp, shielded by a dark blue filter and a yellow filter. As a blank destroy the riboflavin in the original solution by exposing the specimen to a mercury vapor lamp for 1-2 hours, depending upon the concentration. When the fluorescence no longer has a yellowish hue, destruction of the riboflavin is complete. Subtract the blank reading from that of the specimen containing riboflavin.

Micro. The sample will be in isobutanol. Irradiate 1 ml. of solution in a thin-walled Pyrex test tube. Absorb silvery blue fluorescence with a yellow filter. Then expose to ultraviolet for at least an hour to destroy riboflavin and read again as a blank.

Titrametric duplication. Read the fluorescence of the solution against a blank, using a yellow filter with a maximum transmission at 560 mμ, and duplicate the fluorescence of the sample in the blank by titrametric addition of a riboflavin standard. As stock use 5 mg. in 100 ml. of water containing a drop of acetic acid. For use dilute 1:50.

As lumiflavin. Read the fluorescence of the standard and sample against a chloroform blank and calculate the amount in the sample.

In pyridine. The sample is in 20 per cent pyridine plus acetic acid. Add 2 ml. of glacial acetic acid and 1 ml. of 4 per cent potassium permanganate solution. After 2 minutes add 1 ml. of 3 per cent hydrogen peroxide. Filter and read at 470 mμ against water.

Riboflavin Phosphates

The greenish fluorescence of riboflavin is given in neutral solution by riboflavin dinucleotide,[182] the diphosphate, but only to one-ninth the intensity of the monophosphate, the mononucleotide, or free riboflavin.[183]

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[182] Otto Warburg and Walter Christian, *Biochem. Z.* **298**, 150-68 (1938).

[183] Otto A. Bessey, Oliver H. Lowry, and Ruth H. Love, *J. Biol. Chem.* **180**, 755-69, (1949).

The pH must be carefully controlled as the ratio changes. At pH 1.5 the dinucleotide has the same intensity of fluorescence as free riboflavin. The mononucleotide is distinguishable from riboflavin by its distribution between benzyl alcohol and water.[184] Other than by this technic, the nucleotides are released by enzyme action and determined as riboflavin but without distinction as to the form originally present.

Sample—*Tissue. Dinucleotide.* Grind or blend a fresh sample of tissue with 25 volumes of ice water. Mix the sample or an aliquot with an equal volume of ice-cold 20 per cent trichloroacetic acid. -Centrifuge after 15 minutes and, maintaining it at ice temperature, neutralize an aliquot of the clear layer with one-quarter volume of 7 per cent dipotassium phosphate.

Store a second aliquot at 38° for 2 days to hydrolze the mono- and dinucleotides. Then neutralize in the same way. Since in acid solutions the products are more sensitive to light, avoid exposure after neutralization. Use as samples for reading the fluorescence.[185]

Mononucleotide. Shake an aliquot of non-hydrolyzed, neutralized sample with an equal volume of water-saturated redistilled benzyl alcohol. Prepare a diluent of ethanol containing 0.035 per cent of anhydrous sodium acetate and 0.03 per cent of acetic acid. Dilute an aliquot of the benzyl alcohol layer with at least 2 volumes of this as sample to read. Save the extracted aliquot.

Procedure—*Dinucleotide.* Read the difference between the two solutions after reduction (page 307) as riboflavin. The difference is the dinucleotide. Read the benzyl alcohol extract. Based on the fluorescence of dinucleotide = 60 per cent of that of free riboflavin, calculate the mononucleotide. Read the free riboflavin directly. This may be extremely small in tissue.

N-Diethylamino-isopentyl-8-amino-6-methoxyquinoline, Plasmochin, Plasmoquinine, Pamaquine

Plasmochin reacts with diazotized nitroaniline in sulfuric acid to give a red color. Addition of ammonia to a solution in ethanol causes

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184 A. Emmerie, *Rec. trav. chim.* **58**, 290-2 (1939).

185 Helen B. Burch, Otto A. Bessey, and Oliver H. Lowry, *J. Biol. Chem.* **175**, 457-70 (1948).

the color to turn violet.[186] With phosphotungstic-phosphomolybdic acid and sodium carbonate, plasmochin gives the blue color.[187]

Samples—*Tablets.* Extract a weighed sample of ground tablets with absolute ethanol to separate from excipient. Evaporate the extract nearly to dryness. Take up in ethanol, add 10 drops of acetaldehyde, and dilute to a known volume with water, the ethanol to be finally 70 per cent. Use a known volume of the extract as sample for development with diazotized *p*-nitroaniline.

Injectable solutions. Evaporate to dryness and dissolve the residue in 70 per cent ethanol containing 10 drops of acetaldehyde. Develop the color with diazotized *p*-nitroaniline.

Tissues. Grind the minced tissue with sand and 0.2 ml. of 20 per cent sodium hydroxide solution per gram of tissue. Evaporate almost to dryness at room temperature and extract with ether in a Soxhlet for 1.5 hours. Extract the ether extract three times with small amounts of 2 per cent sodium carbonate solution and discard. Remove plasmochin from the combined ether extracts with 5 ml. of 1:5 hydrochloric acid solution. Evaporate the dissolved ether by warming and develop with phosphotungstic-phosphomolybdic acid.

Blood. Render the sample alkaline with 0.1 ml. of 20 per cent sodium hydroxide per ml., shake with several portions of ether, and complete by extraction as for tissues, starting with "Extract the ether extract three times . . . ."

Procedure—*By diazotized p-nitroaniline.* As reagent dissolve 0.5 gram of *p*-nitroaniline in 50 ml. of water and 0.5 ml. of sulfuric acid. To a 7-ml. aliquot add 1 drop of 10 per cent sodium nitrite solution and shake.

Dilute the solution of sample in water containing 10 drops of acetaldehyde per 10 mg. of plasmochin, to 100 ml. with ethanol. Add 0.5 ml. of reagent to 1 ml. of diluted sample. Dilute to a final volume of 2.5 ml. with ethanol and read against a reagent blank. Add a known amount of ammonia to a known volume as a check reading.

With phosphotungstic-phosphomolybdic acid. To 4 ml. of the hydrochloric acid solution of plasmogen add 1 ml. of the phenol reagent (Vol.

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[186] Juan A. Sánchez, *Ann. pharm. franc.* **6**, 495-501 (1949); *Semana med. (Buenos Aires)* **1949, II**, 995-9.

[187] B. K. Nandi and B. B. Dikshit, *Indian J. Med. Research* **25**, 937-50 (1938).

III, page 116). Make strongly alkaline with solid sodium carbonate. Read after 0.5 hour at 520 mμ against a reagent blank.

8-(3-Diethylaminopropylamino)-6-methoxyquinoline, Plasmocid

Plasmocid gives a precipitate with alkaloid reagents and reacts to the usual diazo reagents.[188]

Sample—*Urine.* Add 5 ml. of 50 per cent sodium hydroxide solution to 200 ml. of urine and extract by shaking mechanically with 20 ml. of chloroform for 3 minutes. Remove the chloroform layer and emulsion and extract the residual aqueous layer with 20 ml. of chloroform. Centrifuge the combined solvent and emulsion layers, separating the upper aqueous layer from time to time. Filter the chloroform layer through cotton and wash the filtrate with 0.01 per cent sodium carbonate solution. Extract the plasmocid from chloroform with 1.5, 1.5, and 1 ml. of 1:20 acetic acid. Combine the acid extracts for development.

Procedure—As reagent prepare a 0.25 per cent solution of sulfanilic acid in 1:19 hydrochloric acid. For use mix 1 ml. with 49 ml. of 0.5 per cent sodium nitrite solution. For development mix 1 ml. of sample with 5 ml. of reagent and dilute to 10 ml. with the reagent. Read after 10 minutes against a reagent blank.

6-(2′-amino-1′:6′-dimethylpyridinium-4′-amino)-1:2-dimethylquinolinium dichloride or dimethosulfate, Antrycide

Antrycide is determined in aqueous solution by reaction with colored acids and fluorometrically by reacting with eosine.[189] Most other tertiary and quaternary bases do not give the fluorometric reaction. The monoquaternary analogues containing the pyridine or quinoline ring interfere. The fluorometric method gives results for 0.00001-0.003 mg. of antrycide averaging about 21 per cent low, in terms of amounts added to plasma but accurate to ±2 per cent for 0.00004-0.0004 mg. added to urine.

Sample—*Plasma.* Dilute 1.2 ml. of plasma to 10 ml., add 2 ml. of 15 per cent trichloroacetic acid, and mix. After 10 minutes, centrifuge.

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[188] S. A. Dmitriev, *Farmatsiya* **1940**, No. 1, 9-13; *Med. Parisitol. Parisitic Diseases* (USSR) **9**, No. 1-2, 54-6 (1940).

[189] A. Spinks, *Biochem. J.* **47**, 299-306 (1950).

To 10 ml. of the upper layer add 1 ml. of 4 per cent sodium hydroxide solution. Develop an 11-ml. sample with the eosine reagent.

Urine. Develop a 4-ml. sample with the eosine reagent.

Procedure—*By Congo red.* To 1 ml. of sample containing 0.001-0.01 mg. of antrycide, add 1 ml. of solution containing 0.03 mg. of Congo red and 1 ml. of 0.5 *M* phosphate buffer at pH 7 (Vol. I, page 174). Extract the developed sample with 1 ml. of butanol and read the separated extract against a butanol blank.

By eosine. Prepare a buffered eosine reagent by extracting a solution containing 1 mg. of eosine per ml. of saturated sodium bicarbonate solution. The extracting solvent contains 1 ml. of redistilled *n*-butanol to each 5 ml. of chloroform. Extract with this solvent mixture until no more colored or fluorescent impurities are removed. Dilute the purified eosine solution 1:25 with saturated sodium bicarbonate solution for use with the sample.

To an appropriate sample add 2 ml. of buffered eosine reagent. Shake with 12 ml. of 1:5 butanol-chloroform mixture for 3 minutes and let stand until the lower layer is clear. Remove the lower layer and filter through paper. Read fluorometrically against a blank, using filter PC 9 and letting no light pass below 500 mμ.

2-Ethoxy-6,9-diaminoacridine Lactate, Rivanol

Rivanol is closely related to acriflavine. It gives a deep red color color with sodium nitrite and hydrochloric acid, which can be applied to estimation in urine and in rivanol solutions.[190]

Procedure—To 1 ml. of sample add 1 ml. of 1:5 hydrochloric acid and mix. Add 1 ml. of 6.9 per cent sodium nitrite solution, mix and dilute to 10 ml. Read against a reagent blank.

Chlorodiethylaminopentylaminomethoxyacridine Dihydrochloride, Atabrine, Chinacrin, Mepacrine

Atabrine can be extracted from a strongly alkaline solution. It is obtained in amyl alcohol for acidification and reading[191] or with ether

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[190] M. J. Schulte, *Pharm. Weekblad* 67, 601-4 (1930); I. Maizelis and A. Myasnikova, *Farmatsiya* 4, No. 6/7, 26-30 (1941); Penko Petkov, *Pharm. Acta Helv.* 18 347-60 (1943).

[191] E. B. Vedder and J. M. Masen, *Am. J. Tropical Med.* 11, 217 (1931); C. Tropp and W. Weise, *Arch. exp. Path. u. Pharmakol.* 170, 339-46 (1933); R. C. Wats and B. N. Ghosh, *Records Malaria Survey India* 4, 367-70 (1934); R. N.

and subsequently by extraction into dilute sulfuric acid [192] or hydrochloric acid.[193] In any of these cases a peak can be read at 424 mμ, but a much better one is in the ultraviolet at 280 mμ.[194]

Another method of reading is fluorometrically in organic solvent to ± 10 per cent.[195] Amounts of the order of 0.005 mg. are extracted and estimated. It fluoresces in weakly alkaline solution at 365 mμ. The fluorescence is intensified by caffeine sodium benzoate.[196] Atebrin is also read nephelometrically as the compound with potassium mercuric iodide.[197]

Sample—*Blood.* Absorb the sample in strips of filter paper and dry in an air oven at 50°. Extract the dried filter paper with ether for 2 hours. Evaporate the ether from the extract and dissolve the residue in 4 ml. of 1:120 hydrochloric acid. It usually takes several minutes to get complete solution. Dilute to a known volume. Mix 10 ml. of sample with 0.3 ml. of 20 per cent sodium hydroxide solution and 6 ml. of amyl alcohol. Shake well and separate for acidifying and reading.

For reading fluorometrically, mix 5 ml. of oxalated whole blood with 10 ml. of 5 per cent disodium phosphate solution. Extract with 30 ml. of ether. Discard the aqueous layer. Wash the ether layer with three 5-ml. portions of 0.2 per cent sodium hydroxide solution and then with 5 ml. of water. Now extract the ether layer twice with 5 ml. of 1:360 sulfuric acid. Combine the acid extracts and dilute to 15 ml. as sample for reading the fluorescence.

As an alternative method of extraction, mix 3 ml. of 16 per cent sodium hydroxide solution, 8 ml. of 1:1 isopropanol-isobutanol, 1 ml. of

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Chopra and A. C. Roy, *Ibid.* **70,** 504-5 (1935); R. N. Chopra and A. C. Roy, *Indian J. Med. Research* **24,** 487-8 (1936); *Ibid.* **25,** 455-8 (1937); C. Lataste, Nguyen van Lien, and M. E. Farinaud, *Compt. rend. soc. biol.* **130,** 422-4 (1939).

[192] C. Tropp and W. Weise, *Arch. exp. Path. Pharmakol.* **170,** 339-46 (1933); W. Weise, *Arch. Schiffs-u. Tropen-Hyg.* **41,** 715-20 (1937); Ignazio Traina, *Biochem. Z.* **315,** 111-23 (1943).

[193] J. Carol, *J. Assoc. Official Agr. Chemists* **27,** 360-5 (1944); Earl J. King and Margaret Gilchrist, *Lancet* **1945, I,** 686.

[194] Harold Bissell, H. W. Moeller, and L. D. Seif, *J. Am. Pharm. Assoc.* **34,** 291-2 (1945).

[195] John M. Masen, *J. Biol. Chem.* **148,** 529-39 (1943); Bernard B. Brodie and Sidney Udenfriend, *Ibid.* **151,** 299-317 (1943); M. E. Auerbach and H. William Eckert, *Ibid.* **154,** 597-603 (1944).

[196] G. Ferrari, *Diagnostica tec. lab., (Napoli) Riv. mensile* **5,** 928-32 (1934).

[197] Cleon J. Gentzkow, *Am. J. Trop. Med.* **18,** 149-56 (1938).

water, 7 ml. of petroleum ether, and 5 ml. of blood. Shake mechanically for 5 minutes and decant. Centrifuge the solvent layer and separate from waste aqueous layer. Shake the solvents with 10 ml. of 1.2 per cent sodium hydroxide solution for 1 minute and separate as before. Shake with 10 ml. of 30 per cent isopropanol in 1:120 hydrochloric acid. Centrifuge and read the lower layer by fluorescence.

Urine. Dissolve 10 grams of potassium carbonate in 100 ml. of urine. Shake for 3 minutes with 20 ml. of amyl alcohol. Wash the separated amyl alcohol layer with 10 ml. of saturated aqueous potassium carbonate before acidifying and reading.

For reading fluorescently, mix 50 ml. of urine with 3 ml. of 60 per cent potassium hydroxide solution and extract with 30 ml. and 20 ml. of ether. Wash the combined ether extracts with 15 ml. and 15 ml. of 0.01 per cent sodium carbonate solution. Extract the washed ether extracts with 10 ml. and 10 ml. of 1:360 sulfuric acid. Dilute the acid extract to an appropriate volume.

Tissue. Mince a weighed sample with 30-40 times its weight of water in a blender. Add 2 ml. of 40 per cent sodium hydroxide solution to a 10-ml. aliquot of the suspension and digest for 30 minutes at 80° in a water bath. Continue as for blood for reading fluorometrically from "Extract with 30 ml. of ether."

Procedure—*In amyl alcohol.* Mix 5 ml. of the amyl alcohol solution of atabrin with 1 ml. of glacial acetic acid and read.

By fluorescence. Read the fluorescence at 430 mμ and a carefully controlled temperature.

It is sometimes desirable to intensify the fluorescence of aqueous sulfuric acid extracts. Then as an intensifying reagent dissolve 10 grams of caffeine sodium benzoate in 70 ml. of 56 per cent ethanol. Add 20 ml. of diethanolamine and dilute to 100 ml. with water. To 15 ml. of sample add 1 ml. of the intensifying reagent and mix.

Carbazole

A blue condensation product with salicylic aldehyde is applicable to estimation of up to 6 per cent of carbazole in anthracene with accuracy to ±10 per cent.[198]

Procedure—Dissolve 0.25 gram of sample in 50 ml. of chloroform and use a 5-ml. aliquot. Add 5 ml. of a 0.5 per cent solution of salicylic

[198] N. Strafford and W. V. Stubbings, *Rec. trav. chim.* 67, 918-26 (1948).

aldehyde in glacial acetic acid, and 5 ml. of a 4:1 mixture of glacial acetic acid and concentrated sulfuric acid. After 4 hours at room temperature, dilute to 100 ml. with glacial acetic acid and read at over 600 mμ. Subtract the reading of a blank resulting from similar treatment of pure anthracene.

Prodigiosin

Prodigiosin is a tripyrolmethane dye which can be used as a fine dust to show the fate of inhaled foreign matter such as industrial dusts. The prodigiosin is extracted by organic solvents from physiological specimens and determined by its color in these solvents.[199] Recovery with double extractions, averaged over 90 per cent.

Sample—*Tissue.* Mince aliquot specimens of large organs or entire small organs with 5 ml. of acid chloroform per gram of tissue. Prepare the acid chloroform by mixing chloroform with 1 per cent of ethanol which has been acidified with 3 per cent of glacial acetic acid. Homogenize the tissue, and liquid in a blender for 3 minutes and filter.

Plasma. Centrifuge 5 ml. or more of oxalated blood for 45 minutes. To 2 ml. of the plasma add 1 ml. of 5 per cent sodium hydroxide solution. Add 8 ml. of a 1:1 mixture of petroleum ether and ethanol, and shake for 2 minutes. Stopper the tube and centrifuge for 30 minutes. To the supernatant ether solution add 0.03 ml. of glacial acetic acid.

Procedure—Read the color of the dye in chloroform at 537 mμ, and that in petroleum ether at 533 mμ.

Cyanocobalamin, Vitamin B_{12}

Vitamin B_{12} forms a purple complex of the dicyanide with sodium cyanide in alkaline solution.[200] By hydrolysis with 2:3 hydrochloric acid the vitamin forms 5,6-dimethylbenzimidazole which may be determined either colorimetrically or fluorometrically.[201] Another method is to determine the cyanide released on hydrolysis.

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[199] George V. Taplin, James S. Grevior, Clayton H. Douglas, Arthur Dunn, Camille Finnegan, and Mary Louise Lanier, *J. Am. Pharm. Assoc.* **41**, 510-11 (1952).

[200] John B. Conn, Sara L. Norman, and Thomas G. Wartman, *Science* **113**, 658-9 (1951).

[201] George E. Boxer and James C. Rickards, *Arch. Biochem.* **29**, 75-84 (1950); *Ibid.* **30**, 372-401 (1951).

Procedure—*By cyanide.* In an efficient hood add sufficient solid sodium cyanide to a sample of less than 200 ml. containing in excess of 0.2 mg. of cyanocobolamin to produce a 1 per cent solution. Stir to dissolve and add sufficient 10 per cent sodium hydroxide solution to raise the pH to 9.5-10. In the course of 5 hours standing the product will be the dicyanide complex. Add sufficient sodium sulfate to produce a 20 per cent solution and further solution of sodium hydroxide to raise the pH to 11-11.5. Extract with three successive one-tenth volumes of benzyl alcohol. Discard the aqueous layer.

Combine the benzyl alcohol extracts and add half its volume of chloroform. Make three successive extractions with one-tenth volumes of water. Discard the extracted solvents and dilute the aqueous extracts to 25 ml. Mix a 10-ml. aliquot with 2 ml. of 10 per cent sodium cyanide solution. Adjust another 10-ml. aliquot to pH 5-6 by addition of 2 ml. of 12.5 per cent potassium dihydrogen phosphate solution.

Read the two solutions at 582 mμ and take the difference in readings, using a factor of 1.03 for correction for the extraction.

CHAPTER 7

UREA AND RELATED COMPOUNDS[1]

The members of this family are parents or derivatives of the structures urea, $OC(NH_2)_2$, and guanidine, $HN{:}C(NH_2)$. Not all derivatives of these structures can be collected here for some have that structure as subordinate to a more important one. Thus the placing of creatine here is a choice from placing it as a substituted monobasic acid, even though it carries the basic guanidine structure. Classification is partially on the basis of the reaction of the guanidine structure by cyclization to give creatinine which is an acid only by derivation. The methods are diverse.

Urea, Carbamide, Carbonyldiamide

Urea is estimated by conversion of the urea nitrogen to simple ammonium salts. The ammonia is then developed with Nessler's reagent.[2] Decomposition of urea with the enzyme urease cannot be carried out in glassware which has been used for nesslerization, except after cleaning with nitric acid, since a sorbed film of mercury left on the glass destroys the urease. The maximum activity is obtained at pH 5-5.5. Interference by glucose, creatinine, and other reducing substances can be prevented by adding 0.1 ml. of 0.5 per cent sodium hypochlorite solution to the Nessler's reagent just before mixing with the sample. Addition of potassium persulfate to prevent deterioration of the reagent causes the color to be stable for an hour when read at 500 mμ.[3] As stabilizer, sodium polyanetholsulfonate can be used.[4]

The reaction of biacetyl with urea is used[5] to give accuracy to better than ±3 per cent. Urea under properly controlled conditions gives a

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[1] See Volume III, Chapter 1, for details of organization, condensation, etc.

[2] Harold T. Wrenn, *J. Lab. Clin. Med.* **22**, 1040-5 (1937); Joseph C. Bock, *J. Biol. Chem.* **140**, 519-23 (1941).

[3] Cleon J. Gentzkow and John M. Masen, *J. Biol. Chem.* **143**, 531-44 (1942).

[4] James Hughes and Abraham Saifer, *J. Lab. Clin. Med.* **27**, 391-8 (1941).

[5] James Natelson, Mary Lou Scott, and Charles Beffa, *Am. J. Clin. Path.* **21**, 275-81 (1950).

yellow solution with diacetylmonoxime in strongly acid solution.[6] Subsequent addition of potassium persulfate to destroy hydroxlyamine has been replaced by arsenic acid to destroy it as fast as it is formed.

The red product developed by urea with α-isonitrosopropiophenone in strongly acid solution absorbs at 540 mμ.[7] There is no interference by urinary constituents. Allantoin, citrulline, parabanic acid, phenyl urea, biuret, alloxan, and even proteins give a similar color. The reagent is less volatile than diacetylmonoxime and is less sensitive to citrulline and other urea derivatives. Actually the error from citrulline is negligible.

Urea can be decomposed with a known amount of nitrous acid and the excess determined with sulfanilic acid and phenol.[8] Amino acids and ammonia do not interfere. The method will determine up to 2 mg. of urea per ml. Air must be excluded from the acid solution containing nitrite to avoid oxidation of the reagent. Glycine, alanine, and leucine do not interfere.

Diazotized sulfanilic acid gives a yellowish green with urea.[9] The color is not given by ammonium salts, guanidine, creatine, creatinine, or amino acids.

Urea reacts with furfural in the presence of stannous chloride to give a purple. On addition of sodium acetate this is modified to brown or brownish green.[10] When dissolved in sulfuric acid and oxidized with

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[6] W. R. Fearon, *Biochem. J.* 33, 902-7 (1939); Andrew A. Ormsby, *J. Biol. Chem.* 146, 595-604 (1942); S. B. Barker, *Ibid.* 152, 453-63 (1944); Einhart Kawaran, *Sci. Proc. Roy. Dublin Soc.* 24, 63-70 (1946); S. Natelson, M. L. Scott and C. Beffe, *Am. J. Clin. Pathol.* 21, 275-81 (1951); Howard S. Friedman, *Anal. Chem.* 25, 662-4 (1953).

[7] F. Leuthardt and B. Glasson, *Helv. Chim. Acta* 25, 630-5 (1942); F. Leuthardt, *Z. physiol. Chem.* 252, 238-60 (1938); Reginald M. Archibald, P. Ortiz, E. Stroh, and J. Bronner, *J. Biol. Chem.* 157, 507-18 (1945); Jarlyn O. Halverson and M. O. Schultze, *Ibid.* 186, 471-6 (1950).

[8] Juan A. Sánchez, *Semana méd.* (*Buenos Aires*) 1935, II, 503-7; *Rev. centro estud. farm. bioquím.* 25, 364-72 (1935); *J. pharm. chim.* [8] 23, 188-99 (1936); W. Brandt, *Mikrochemie* 22, 181-6 (1937).

[9] O. Weltmann and H. K. Barrenscheen, *Klin. Wochenschr.* 1922, 1100; Setsuro Aoi, *Nagoya J. Med. Sci.* 3, 13-8 (1928).

[10] E. Obermer and R. Milton, *Diagnostica tec. lab.* (*Napoli*) *Riv. mensile* 5, 741-52 (1934).

hydrogen peroxide, a blue color is developed with phosphotungstic-phosphomolybdic acid.[11] Other variations include development with phloroglucinol [12] and reading the opacity of a suspension.[13]

Urea is quantitatively precipitated as dixanthylurea by xanthydrol in the absence of protein.[14] The precipitate when dissolved in nitric acid is yellow with a green fluorescence suitable for reading at 470 mμ.[15] The precipitate is also dissolved in 1:1 sulfuric acid for reading,[16] at 445 mμ. The only interference by normal blood constituents is by very high concentrations of allantoin. Thiourea interferes.

Samples—*Blood or serum.*[17] *Enzyme hydrolysis.* As buffer use 0.5 per cent sodium acetate trihydrate solution in 1:350 acetic acid. Mix 1.4 ml. of this with 0.2 ml. of serum and add powdered urease or a few drops of 1 per cent aqueous urease solution.[18] Jack-bean powder can also be applied directly.[19] Incubate at 50-55° for about 5 minutes and cool, or let stand for 1 hour at 25°.[20] Add 0.2 ml. of 10 per cent sodium tungstate solution. Add 0.2 ml. of 1:54 sulfuric acid, mix well, and centrifuge after 5 minutes. Mix 0.5 ml. of the upper layer, 0.2 ml. of 1:3 sulfuric acid, and 0.5 ml. of 20 per cent sodium hydroxide solution. Dilute to 10 ml. with water for development with Nessler's reagent.

Alternative buffers for dilution are a mixture of 19 parts of 14.7 per cent solution of sodium citrate dihydrate and 1 part of 7.4 per cent

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11 Florence Beattie, *Biochem. J.* **22**, 711-2 (1928); Shun-Ichi Yoshimatsu, *Tohoku J. Exptl. Med.* **13**, 1-5 (1929); F. Böhm and G. Grüner, *Biochem. Z.* **287**, 65-70 (1936).

12 Mario Zappacosta, *Diagnostica tec. lab.* (*Napoli*) *Riv. mensile* **6**, 388-96 (1935).

13 M. Viré, *Bull. soc. chim. biol.* **22**, 185-91 (1940).

14 R. Fosse, *Compt. rend.* **158**, 1076-9 (1914); *Ibid.* **159**, 253-6 (1914); R. Fosse, A. Robyn and F. Francois, *Ibid.* **159**, 367-9 (1914); M. Nicloux and G. Welter, *Ibid.* **173**, 1490-3 (1921).

15 A. Gigon and M. Noverraz, *Schweiz. med. Wochschr.* **70**, 464-5 (1940).

16 Margaret H. Lee and Elsie M. Widdowson, *Biochem. J.* **31**, 2035-45 (1937); E. E. Martinson and I. V. Fetisenko, *Lab. Prakt.* (USSR) **1939**, No. 5, 19-21; Mildred G. Engel and Frank L. Engel, *J. Biol. Chem.* **167**, 535-41 (1947); F. Faure, *Ann. biol. clin.* (Paris), **11**, 63-6 (1953).

17 Peter Balint, Antel Kincses, and Imre Zsiga, *Orvosi Hetilap.* **89**, 343-5 (1948).

18 V. Everett Kinsey and Phyllis Robison, *J. Biol. Chem.* **162**, 325-31 (1946).

19 H. N. Naumann, *J. Lab. Clin. Med.* **26**, 405-8 (1940-1).

20 Joseph C. Bock, *Ibid.* **27**, 1222-4 (1942).

citric acid solution;[21] 15 per cent potassium acetate in 1 per cent acetic acid;[22] or 8.2 per cent orthophosphoric acid.[23]

Removal of traces of protein. A trace of protein left by the tungstic acid precipitation of proteins can cause a cloudy nesslerized solution. To 2 ml. of blood in a small flask add 0.3 ml. of 1 per cent aqueous urease solution and 2 drops of buffer containing 14 per cent of sodium pyrophosphate and 2 per cent of pyrophosphoric acid. After 15 minutes at 50° add 13 ml. of water, 2 ml. of 10 per cent sodium tungstate solution, and 2 ml. of 1:54 sulfuric acid. Mix, let stand to coagulate, and filter.

Mix 10 ml. of the tungstic acid filtrate with 0.5 ml. of a 20 per cent solution of sodium hydroxide, a drop of the phosphate buffer, and 2 drops of a 10 per cent solution of calcium nitrate. Stir and centrifuge for 2 minutes. The precipitated calcium phosphate removes the last trace of proteins. Decant the supernatant liquid. Wash down with 2 ml. of 1 per cent sodium hydroxide solution, mix, and centrifuge for 2 minutes. Decant into the first portion of supernatant liquid, dilute to 20 ml., and develop with Nessler's reagent.

Acid hydrolysis. Mix 1 ml. of blood with 7 ml. of water and add 1 ml. of 10 per cent sodium tungstate solution. Mix and add 1 ml. of 1:54 sulfuric acid. The color gradually changes from a pink to a dark brown. If this change is incomplete it indicates that too much citrate or oxalate was added to the blood as anticoagulant. In that case add 1:18 sulfuric acid drop by drop, shaking vigorously after each addition until coagulation is complete. Wet a filter paper with the coagulated blood. Pour the mixture on this filter and if the filtrate is not clear return portions to the funnel until it comes through crystal-clear. The sulfuric acid should be sufficient in amount to set free all the tungstic acid from the sodium tungstate and allow about 10 per cent excess. The tungstic acid filtrate should not give a strongly positive acid reaction with Congo red paper or the results will be low.

Add 1 ml. of 1:35 sulfuric acid to 5 ml. of blood filtrate and heat in an autoclave at 150° for 10 minutes. Let cool to 100° before opening the autoclave. Place 2 ml. of 1:250 hydrochloric acid in a receiver. The delivery tube must dip just below the surface. Add 2 ml. of a 10 per cent solution of sodium carbonate, a glass bead, and 1 drop of paraffin

[21] Osvaldo V. Mostro and Alfredo C. Romano, *Anales. farm. bioquim.* (Buenos Aires) **16**, 52-60 (1945).

[22] S. K. Tower, *Chemist-Analyst* **32**, 7-8 (1943).

[23] Andre C. Kibrick and Sol Krupp, *Proc. Soc. Exptl. Biol. Med.* **73**, 412-3 (1950).

oil to the solution of hydrolyzed sample. Distil with a micro burner at such a rate that no steam will escape from the receiver, for 3 minutes. Then disconnect the receiver, touch the delivery tube to the side of the receiver, and continue to distil for one minute longer. Rinse off the outside of the delivery tube and cool. Dilute the distillate to 20 ml. for development with Nessler's reagent.

For development of serum with diacetyl, prepare a tungstic acid reagent by mixing equal volumes of 2.2 per cent solution of sodium tungstate dihydrate and 1:240 sulfuric acid. Mix 0.1 ml. of serum with 1.9 ml. of this reagent and shake. Centrifuge after 2 minutes.

For development with diacetylmonoxime, mix 10 ml. of water and 1 ml. of whole blood. Add 7 ml. of barium hydroxide solution previously titrated to establish that 13.5-14.5 ml. will titrate 4 ml. of 5 per cent zinc sulfate solution (cf. Vol. III, Page 61). After 30 seconds add 2 ml. of 5 per cent zinc sulfate solution. Shake vigorously and filter. Develop with diacetylmonoxime.

For development with α-isonitrosopropiophenone, deproteinize with zinc sulfate and barium hydroxide, finally having a 1:10 dilution. For development after precipitation as dixanthylurea, use a 1:10 blood filtrate, prepared with trichloroacetic acid.[24] For development with nitrous acid use a 1:10 phosphotungstic acid filtrate or a trichloracetic acid filtrate. For development with diazotized sulfanilic acid use a trichloroacetic acid filtrate.

Urine. Enzyme hydrolysis. As buffer prepare 0.5 per cent sodium acetate trihydrate solution in 1:150 acetic acid. Wash zeolite with water then 2 per cent acetic acid, and then water. To the moist solid add 1 ml. of 1:10 urine and 1 ml. of the diluted buffer. Dilute to 10 ml. with water, shake vigorously for 5 minutes, and sediment. Mix 1 ml. with 2 ml. of diluted buffer and some urease. Incubate at 50-55° for about 5 minutes. Cool and add 0.5 ml. of 10 per cent sodium tungstate solution and 0.5 ml. of 1:54 sulfuric acid. Shake and after 5 minutes centrifuge. Mix 1 ml. with 0.2 ml. of 1:3 sulfuric acid and 0.5 ml. of 20 per cent sodium hydroxide solution. Dilute to 10 ml. and develop with Nessler's reagent.

For more efficient protein removal, mix 1 ml. of 1:9 urine and 0.3 ml. of urease solution. Add 2 drops of phosphate buffer mixture containing 14 per cent of sodium pyrophosphate and 2 per cent of pyrophosphoric acid, and heat at 50° for 15 minutes. Remove and add 8 ml.

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[24] M. Viré, *Bull. soc. chim. biol.* **22,** 185-91 (1940).

of water, 1 ml. of a 10 per cent sodium hydroxide solution, and 2 drops of a 10 per cent calcium nitrate solution. Stir and centrifuge for 1 minute. Decant the clear liquid from the precipitated calcium phosphate, add 10 ml. of a 1 per cent sodium hydroxide solution to the precipitate, stir, and centrifuge again. Decant the supernatant liquid into the previous decantate, dilute to 50 ml., and develop an aliquot with Nessler's reagent.

For development with diacetylmonoxine, adjust 2 ml. to pH 4-5 with 2 drops of 1:50 acetic acid. Boil and filter. Dilute to 500 ml. for use of aliquots.

For development with α-isonitrosopropiophenone, it may be necessary to deproteinize. If required add to 5 ml. an equal volume of buffer containing 1 per cent of sodium acetate trihydrate and 1 per cent of acetic acid. At this pH of 4-5 heat to 100° and centrifuge or filter. Then dilute as necessary.

For development with diazotized sulfanilic acid, mix 8 ml. of urine with 2 ml. of saturated basic lead acetate solution and filter. Add 3 ml. of saturated sodium sulfate solution to 5 ml. of filtrate and filter. Dilute 2.5 ml. of this filtrate to 25 ml.

Procedure—*By Nessler's reagent.* To prepare the reagent, dissolve 15 grams of potassium iodide in 10 ml. of warm water. Add 20 grams of mercuric iodide and stir until dissolved. Dilute to about 100 ml. and filter. Dilute to about 200 ml. and filter again after a few days. Prepare a 10 per cent sodium hydroxide solution by dilution of a saturated solution. Add 75 ml. of the potassium mercuric iodide solution and 75 ml. of water to 350 ml. of 10 per cent sodium hydroxide solution. The formula of Nessler's reagent used makes no significant difference.[25] Add 0.5 ml. of Nessler's reagent for each 4 ml. of sample to be developed and read at 420 mμ or compare with standards (Vol. II, page 817).

By α-isonitrosopropiophenone. To 2 ml. of sample add 5 ml. of water and 5 ml. of a mixture of 3 volumes of 85 per cent phosphoric acid and 2 volumes of 1:1 sulfuric acid. Add 0.4 ml. of 4 per cent α-isonitrosopropiophenone in ethanol. Mix and heat in boiling water in the absence of light. After exactly 1 hour cool to room temperature, also in the dark, and read after 15 minutes at 540 mμ against a reagent blank. If much allantoin is present reduce the heating time to 20 minutes, which

[25] A. D. Marenzi, *Anales farm. bioquím.* (Buenos Aires) **16**, 3-28 (1945).

will develop 27 per cent as much color from urea but only 4 per cent as much from allantoin.

By diacetyl. To 1 ml. of the sample, add 1 ml. of 5 per cent solution of diacetyl in ethanol and heat in boiling water for 10 minutes. Cool and read at 440 mμ against a reagent blank.

By diacetylmonoxime. As arsenic reagent dissolve 100 grams of arsenic acid in 1 liter of 1:1 sulfuric acid at 50°. To 1 ml. of sample add 0.5 ml. of a 1 per cent solution of diacetylmonoxime in 1:19 acetic acid. Add 1 ml. of the arsenic reagent and heat at 100° for exactly 10 minutes. Let cool for 3 minutes and dilute to 10 ml. Read at 420 mμ within 7 minutes against a reagent blank.

By nitrous acid. Mix 5 ml. of sample with 1 ml. of a 0.01 per cent solution of sodium nitrite. Dilute to 10 ml. and cover with a layer of white mineral oil. Add 30 drops of concentrated sulfuric acid, mix well, and heat at 65° for 25 minutes. Prepare a reagent to contain 0.5 gram of sulfanilic acid and 0.75 gram of phenol per 100 ml. of 1:40 sulfuric acid. Add 1 ml. of this reagent, mix well, and add 4 ml. of concentrated ammonium hydroxide. Mix and let stand for 5 minutes before reading against a reagent blank.

By diazotized sulfanilic acid. As a fresh reagent, mix 2 volumes of a cold 0.5 per cent solution of sulfanilic acid in 1:19 hydrochloric acid, and 1 volume of a 0.2 per cent solution of sodium nitrite in water. To 1 ml. of sample add 2 drops of the reagent. Read against a sample blank.

By furfural. Mix 0.2 ml. of sample with 1 ml. of 10 per cent stannous chloride in concentrated hydrochloric acid. Add 0.3 ml. of colorless furfural in 7 ml. of glacial acetic acid. Dilute to 21 ml. with 5 per cent gum ghatti solution. After 30 minutes to develop a purple color, add 3 ml. of 30 per cent sodium acetate solution and dilute to 25 ml. with the gum ghatti solution. Mix and read after 30 minutes against a reagent blank.

As dixanthylurea in sulfuric acid. Mix 1 ml. of protein-free sample, 1 ml. of acetic acid, and 0.2 ml. of 5 per cent solution of xanthydrol in 4:1 acetic acid-methanol. After refrigerating overnight, centrifuge and decant. Wash the precipitate with 4 ml. of a saturated aqueous solution of dixanthylurea, centrifuge, and decant. Repeat the washing with 4 ml. of saturated solution of dixanthylurea in methanol. Dissolve the

precipitate in 5 ml. of 1:1 sulfuric acid and read at 420 mμ against 1:1 sulfuric acid.

As dixanthylurea by phosphotungstic-phosphomolybdic acid. Follow the preceding technic through ". . . dixanthylurea in methanol." Dissolve the precipitate in 1 ml. of 1:1 sulfuric acid by warming. Dilute with a few ml. of water and add 1 ml. of 3 per cent hydrogen peroxide. Immerse in a boiling water bath for 5 minutes. Add 5 ml. of phosphotungstic-phosphomolybdic acid reagent (Vol. III page 116) and 10 ml. of 20 per cent sodium carbonate solution. Heat in the boiling water bath for 3 minutes, cool, and dilute to 25 ml. Read at 430 mμ.

Iminourea, Carbamidine, Guanidine

Guanidine gives a deep red color tending toward orange with a reagent containing sodium nitroprusside, potassium ferrocyanide, and hydrogen peroxide in alkaline solution.[26] Methyl guanidine gives the same reaction but with a trend toward the purple. The reagent itself is light yellowish green. Creatine and creatinine give a faint coloration due to liberation of methyl guanidine, but do not interfere materially. Uric acid and ammonia lessen the color development. Urea produces a color about one-tenth as intense as that of guanidine. Ethanol gives a faint reaction. Methyl urea, β-methyl hydantoin, β-methyl hydantoic acid, and glucose produce no color with the reagent.

When the hydrogen peroxide is omitted, the conditions of development change somewhat.[27] The color is orange to red. This reagent produces 92 per cent as much color with asymmetric dimethylguanidine as with methylguanidine. Guanidine produces only about 40 per cent as much color as does methylguanidine. This reagent is very sensitive to salts, necessitating separation by sorption. It reacts with arginine to give a rose-red color and with histidine and cysteine to give a green color. Cystine gives no color. Adrenaline, physostigmine, pilocarpine, and pelletierine give a red color. Procaine gives a green color. Methyl urea, β-methyl hydantoin, β-methyl hydantoic acid, sodium glycocholate, and thioneine do not give a color.

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[26] H. R. Marston, *Australian J. Exptl. Biol. Med. Sci.* **1**, 99-103 (1924); *Ibid.* **2**, 57-9 (1925).

[27] J. J. Pfiffner and V. C. Myers, *Proc. Soc. Exptl. Biol. Med.* **23**, 830-2 (1926); J. J. Pfiffner and V. C. Myers, *J. Biol. Chem.* **87**, 345-55 (1930); Jerome E. Andes and Victor C. Myers, *J. Lab. Clin. Med.* **22**, 1147-54 (1937); *J. Biol. Chem.* **118**, 137-45 (1937); Paulo da Mota Lira, *Rev. quim. farm.* **11**, No. 2, 21-31; No. 3, 9-18 (1946)).

When a solution of guanidine is treated with 1,2-naphthoquinone-4-sodium sulfonate and further developed, the resulting bright red color is a quantitative measure of the guanidine present.[28] The color is due to formation of 4-guanidonaphthoquinone-1,2. This reaction detects 0.05 mg. of guanidine per ml. With 0.5-1 mg. a red-brown precipitate forms in aqueous solution but is soluble in ethanol. In solutions of mixed amino acids the amount of reagent must be increased. Urea is added to stabilize the color. Hydrochloric acid prevents formation of a red color by methyl guanidine but a yellow is produced. Aminoguanidine gives a red which quickly fades to yellow.

There is no interference by 1 mg. per ml. of glycocyamine, glycocyamidine, creatine, creatinine, hydantoic acid, arginine, biuret, xanthine, methyl xanthine, adenine, guanine, uric acid, allantoin, alloxan, glycine, alanine, phenylalanine, aspartic acid, valine, cysteine, cystine, glutamic acid, leucine, proline, tryptophan, histidine, histamine, lysine, thymine, uracil, urea, thiourea, acetone, pyruvic acid, catechol, epinephrine, dihydroxyphenylalanine, hydrazine, glucose, parabanic acid, or urethane. Large amounts of ammonia, methylamine, indole, and benzidine interfere. Benzidine can be eliminated by precipitation as the sulfate and filtering. Indole is readily volatilized by heating in slightly acid solution. Ammonia and methylamine rarely occur in biological solutions in amounts which will interfere. In case they do, the guanidine is separated by picric acid precipitation.

A bluish to yellow-green turbidity is produced in solutions containing guanidine, on the addition of a special form of Nessler's reagent.[29] Phosphotungstic acid must be absent.

Sample—*Blood.* Destroy urea in 10 ml. of blood by urease and buffer solution (page 319). Precipitate proteins with phosphotungstic acid as follows immediately thereafter, and filter. Add 1 ml. of a saturated sodium carbonate solution to the filtrate and evaporate to dryness. Extract the residue with 3 successive 10-ml. portions of absolute ethanol. Filter, evaporate to dryness, and take up with 4 ml. of water for development with nitroprusside-ferrocyanide-peroxide.

For development with nitroprusside-ferrocyanide reagent, mix 50 ml. of deproteinized blood filtrate and 4 drops of 10 per cent sodium hydrox-

[28] M. X. Sullivan, *Proc. Soc. Exptl. Biol. Med.* 33, 106-8 (1935); *J. Biol. Chem.* 110, 233-5 (1936); M. X. Sullivan and W. C. Hess, *J. Am. Chem. Soc.* 58, 47-8 (1936).

[29] R. Rittmann, *Biochem. Z.* 172, 36-9 (1926).

ide solution. Add 0.5 gram of purified blood charcoal. This sorbs all of the guanidine and derivatives and some of the creatine. Shake and, after exactly 2 minutes, filter by suction. Wash with a few ml. of 0.3 per cent sodium carbonate solution. Treat the filter paper and charcoal with 25 ml. of 1:500 dilution of hydrochloric acid with ethanol. Evaporate to dryness at 80-90°. Add 25 ml. more of the acid ethanol and filter after a few minutes. Evaporate 20 ml. of filtrate to dryness at 80-90°. Add 2 ml. of 1:60 hydrochloric acid to the residue and autoclave at 120° to convert creatine to creatinine. Evaporate to dryness at 80°, add 2 ml. of absolute ethanol, and again evaporate to remove the last traces of hydrochloric acid. Take up the residue in 2 ml. of water as the sample.

Procedure—*By nitroprusside-ferrocyanide-peroxide.* Prepare a stock solution of the reagent by dissolving 6 grams of sodium nitroprusside and 8.5 grams of potassium ferrocyanide in water and diluting to 100 ml. About 15 minutes before using, mix 1 volume of this solution with 1 volume of 10 per cent sodium hydroxide solution and 2 volumes of 3 per cent hydrogen peroxide. Add 1 ml. of the freshly prepared reagent to 4 ml. of sample. Read between 10 and 60 minutes later against a reagent blank.

By nitroprusside-ferrocyanide. To 2 ml. of sample add 0.5 ml. of a reagent containing 0.9 per cent each of sodium hydroxide, sodium nitroprusside, and potassium ferrocyanide. Read 7-8 minutes after mixing against a reagent blank.

By 1,2-naphthoquinone-4-sodium sulfonate. Mix 1 ml. of sample and 2 ml. of a 1 per cent aqueous solution of 1,2-naphthoquinone-4-sodium sulfonate. Add 0.5 ml. of 4 per cent sodium hydroxide solution. Heat in boiling water for 1 minute and cool. Add successively with mixing 0.5 ml. of 25 per cent urea solution, 0.5 ml. of concentrated hydrochloric acid, 1 ml. of concentrated nitric acid, and 5 ml. of ethanol. Read against a reagent blank.

Nephelometrically by Nessler's reagent. To prepare the Nessler's reagent, add a saturated solution of mercuric chloride to a solution of 15.5 grams of potassium iodide in 62.5 ml. of water, until a precipitate appears. Dissolve this by adding a few drops of potassium iodide solution. Add more mercuric chloride to produce a very slight but permanent precipitate. Add a solution of 37 grams of potassium hydroxide in 37.5 ml. of water and dilute to 250 ml. Let stand in the dark for 24-48

hours, decant off the supernatant liquid, and keep in a cool dark place. It keeps from 8 to 21 days.

Dilute the sample to 10 ml. with water and add 5 ml. of the special Nessler's reagent very slowly and with constant stirring. Dilute to 50 ml. and read nephelometrically.

Methylguanidine

The properties of methylguanidine are very similar to those of guanidine. It reacts with a mixture of sodium nitroprusside, potassium ferrocyanide, and hydrogen peroxide much as guanidine does but gives a red color tending toward purple. For details of the procedure, see guanidine (page 326). Likewise it reacts with the reagent from which hydrogen peroxide has been omitted (page 326).

A distinctive reaction of methylguanidine is that with α-naphthol and sodium hypobromite to give a red color.[30] Methyl guanidines are separated from deproteinized blood filtrate by activated carbon and extraction from this with ethanol. The reaction is not given by guanidine, nitroguanidine, glycocyamidine, creatine or creatinine, hydantoic or thiohydantoic acid, biuret, or nitroarginine. The reaction is given by α-guanidine butyric acid and presumably by other guamido acids.

Sample—*Blood.* To 50 ml. of 1:5 blood filtrate deproteinized with sodium tungstate and sulfuric acid (page 319) add 5 drops of 10 per cent sodium hydroxide solution and mix. Add 0.5 gram of purified activated carbon and mix. After shaking for a few minutes pour on a wet filter paper. Treat the paper containing the wet precipitate with 25 ml. of 1:600 hydrochloric acid in ethanol. Mix and let stand for 12 hours. Filter and evaporate 20 ml. of filtrate to dryness at 80-90°. Extract the residue with 10, 5, and 5 ml. of absolute ethanol. Centrifuge the extract and evaporate the clear decantate. Dissolve the residue in 5 ml. of 2 per cent sodium hydroxide solution.

Procedure—To 5 ml. of sample add 1 ml. of 10 per cent sodium hydroxide solution. Mix and add 1 ml. of a reagent freshly prepared by dilution of 1 part of 0.1 per cent α-naphthol solution in ethanol with 4 parts of water. Place in cracked ice with about 8 ml. of a 40 per cent solution of urea. After 1 hour add 0.4 ml. of sodium hypobromite pre-

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[30] M. Zappacosta, *Bull. soc. ital. biol. sper.* **10**, 705-8 (1935); *Diagnostica tec. lab. (Napoli) Riv. mensile* **6**, 441-60 (1935).

pared by solution of 2.5 grams of bromine in 100 ml. of 5 per cent sodium hydroxide solution with cooling. Mix and after 15 seconds add 1 ml. of the cold 40 per cent urea solution. Read at 6-8 minutes as fading to yellow begins after that time.

Methylguanylglycine, Creatine, and Methylglycocyamidine, Creatinine

Creatine and creatinine necessarily are considered together. Creatine is $HN{:}C{-}N(CH_3)CH_2CO_2H$ which by cyclization in forming the acid

$$\begin{array}{l} HN{:}C{-}N(CH_3)CH_2CO_2H \\ \quad\;\; | \\ \quad NH_2 \end{array}$$

amide forms creatinine $HN{:}C{-}N(CH_3)CH_2CO$ (the C bonded through HN to the CO, closing the ring). So creatine is converted to creatinine by autoclaving at 20 pounds for 40 minutes.[31] Both creatine and creatinine are destroyed by heating for 2 hours at 80° in the presence of 8 per cent sodium hydroxide, a convenient method to get a sample blank.[32]

The red color of creatinine with picric acid in alkaline solution is stable for at least 30 minutes and is suitable for reading.[33] The color is probably that of a tautomer of creatinine picrate. The maximum absorption is at 520 mμ, but the corresponding maximum of the reagent is at 500 mμ. Reading at 603 mμ takes it even further from the maximum of the reagent.[34] Amino acids do not interfere.[35] Acetyl acetone causes high results if the determination is made at once, but later gives low values. Guanidine carbonate and dimethylguanidine hydrochloride diminish the color.[36] Aldehydes, polyphenols, and glucuronic acid increase it. Temperature has no significant effect on the color over a reasonable range.[37] Probably the best technic for minimizing interference is to read at 60 minutes and 120 minutes and extrapolate back to zero time.[38]

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[31] Barnett Sure and Violet M. Wilder, *J. Lab. Clin. Med.* **26**, 874-8 (1941).
[32] Maurice Gilly, *Compt. rend.* **226**, 605-6 (1948).
[33] M. Jaffe, *Z. physiol. Chem.* **10**, 391-400 (1886).
[34] J. V. Kostir and J. Sonka, *Biochem. et Biophys. Acta* **8**, 86-9 (1952).
[35] Josef Schormuller and Hans Mohr, *Z. Untersuch. Lebensm.* **75**, 97-118 (1938).
[36] K. Dirr and H. L. Schade, *Z. Ges. exptl. Med.* **100**, 20-37 (1937).
[37] Tomitarô Fukuyama, *Igaka to Seibutsugaku* **18**, 181-3 (1951).
[38] Henry D. Lauson, *J. Applied Physiol.* **4**, 227-44 (1951).

Alkaline salts of aminodinitrophenol and diaminonitrophenol show nearly the same color properties as the creatinine solution. The reaction is complete in the presence of 2.5 moles of picric acid to each one of creatinine. By acidifying the alkaline mixture within 10 minutes, picric acid and creatinine are recovered quantitatively. After 30 minutes, when fading occurs, picric acid can no longer be recovered quantitatively. In its action on the alkaline salt of picric acid creatinine therefore appears to behave as a very powerful reducing agent.

The conversion of creatine to creatinine is 97-98 per cent complete in the presence of picric acid but substantially complete if the reagent is not added first.[39] One method is to compare the color with that of a solution of potassium bichromate under carefully fixed conditions.[40] Another artificial standard consists of 3 grams of cerium sulfate, 60 ml. of concentrated nitric acid, and 36 ml. of water, boiled and let stand for 24 hours.[41] Stability is controlled by addition of very dilute sodium thiosulfate.

A more satisfactory method is to read at 520 mμ.[42] Beer's law holds only approximately. In the presence of sugars the coloration should be developed at 0° or the reaction of sugars and picric acid will interfere. Natural esters such as *n*-methylhydantoin give the color reaction. Treatment of blood filtrates with hot sodium hydroxide solution forms hydrolytic products which give the reaction. Treatment of blood filtrates or creatinine solutions with hot formaldehyde destroys the reaction. Blood deproteinized with phosphotungstic acid gives lower values than if deproteinized by precipitation of zinc sulfate with barium hydroxide.[43]

Creatinine in alkaline solution gives a brilliant garnet-red to purple with sodium 3,5-dinitrobenzoate,[44] suitable for estimation. The reagent solution is a pale yellow which does not interfere when more than 0.025

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39 G. Frederick Lambert, *J. Biol. Chem.* **161**, 679-83 (1945).

40 Emanuel E. Mandel, Edward B. Lehmann, Robert Dorin, and Lorrain Schmelzle, *J. Lab. Clin. Med.* **34**, 720-4 (1949).

41 Ferenc Solti, *Kísérletes Orvostudomány* **1**, 126-7 (1949).

42 T. Addis, Evelyn Barrett, and Jean T. Menzies, *J. Clin. Invest.* **26**, 879-82 (1947).

43 E. C. Noyons, *Chem. Weekblad.* **36**, 384-90 (1939).

44 Adolph Bolliger, *J. Proc. Roy. Soc. N. S. Wales* **69**, 224-7 (1936); Stanley R. Benedict and Jeanette A. Behre, *J. Biol. Chem.* **114**, 515-32 (1936); Wilson D. Langley and Margaret Evans, *Ibid.* **115**, 333-41 (1936); A. Franceschi, *Farm. Glasnik* **7**, 347-53 (1951).

mg. of creatinine is present. Less color is produced with glucose or acetoacetic acid than by picrate reagent. No color is given by this reagent with glucose, acetone,[45] acetoacetic acid, creatine, arginine, methylguanidine, *as*-dimethylguandine, guanidine, fructose, cystine, benzal creatinine, acetylbenzal creatinine, tribenzoyl creatinine, creatine oxime, or glycocyamine. The reagent is somewhat sensitive to uric acid, furfural, and formaldehyde. An amount of uric acid ten times that normally present in urine does not interfere Reaction is given by methyl-, dimethyl- and ethyl creatinine hydroiodide, 4- and 5-benzoyl creatinine, 5-benzyl creatinine hydrochloride, 2-benzyl creatine, glycocyamidine hydrochloride and hydantoin. Such agents as zinc and hydrogen sulfide reduce the reagent.

Pure solutions give an error of less than ±1 per cent in the range 0.6-1.6 mg. of creatinine per ml. of sample. The values are not proportional to concentration if less than 0.03 mg. is present in a 10-ml. sample. The instability of creatinine in alkaline solution renders the resulting color unstable after a short time. Addition of sodium acetate buffer to the reagent renders the color more stable.[46]

The general reaction of diacetyl with amines is, for acetyl benzoyl, limited to a color in alkaline solution with guanidine derivatives having at least one free NH_2 group. The reaction is suitable for direct estimation of creatine by its violet color.[47] Prompt addition of hydroxylamine after cooling prevents destruction of color by air oxidation. The color fades gradually, but the rate of fading is the same for sample and standard.

An analogous reaction is that of diacetyl, creatine, and a phenol such as α-naphthol.[48] Creatinine or creatinine-phosphate do not interfere and arginine, guanidine, and glycocyamine give but little more than 10 per cent of the color of creatine. An appropriate urine sample is 1:10 or 1:50 dilution.

A method of estimation of creatinine is by addition of an excess of potassium mercuric thiocyanate. The mercuric complex and the creatinine combine. The excess mercury is then determined by the

[45] A. Colangiuli and R. Breda, *Diagnostica tec. lab., Riv. mensile* 12, 182-90 (1941).

[46] E. Lehnantz, *Z. physiol. Chem.* 271, 265-74 (1941).

[47] Konrad Lang, *Ibid.* 208, 273-80 (1932).

[48] P. Eggleton, S. R. Elsden, and Nancy Gough, *Biochem. J.* 37, 526-9 (1943); J. Raaflaub and I. Abelin, *Biochem. Z.* 321, 158-65 (1950).

dithizone method.[49, 50] The method is applicable to 0.001-0.02 mg. of creatine and there is no interference by guanidine, methylguanidine, uric acid, ammonia, or 30-times the concentration of creatine. A similar method is carried out with mercuric nitrate in aqueous sodium bicarbonate.[51] Conversely, creatine may be used to form molybdenum blue by reduction.[52]

Color is developed with purified creatinine by oxidation with mercuric oxide in alkaline solution to methylguanidine and development with hypobromite and α-naphthol.[53] Reduction of mercuric chloride or mercuric acetate by creatinine in alkaline solution gives mercurous chloride, conveniently estimated as mercuric sulfide.[54] There is interference by acetoacetic acid, acetone, ascorbic acid, and glucose. All but glucose can be eliminated.

Modified Nessler's reagent, consisting of 1 per cent of potassium mercuric iodide, 18 per cent of sodium hydroxide, and 7.5 per cent of potassium iodide, is much less reactive to ammonia and more reactive to creatinine.[55] There is no interference by creatine, guanidine, methyl guanidine, sarcosine, uric acid, or even by ammonia itself.

Procedure—*By picric acid. Creatinine. Urine.* Mix 1 ml. of urine with 20 ml. of 1.2 per cent picric acid solution and add 1.5 ml. of a 10 per cent solution of sodium hydroxide. Mix and after 5 minutes dilute to 100 ml. and read at 520 mμ against a reagent blank. It is often desirable to dilute to a known extent to a specific gravity of 1.01.[56]

Alternatively mix the urine with one-half volume each of 1:150 hydrochloric acid and 5 per cent aqueous phosphotungstic acid. Filter, dilute to 100 volumes, and mix 5 ml. with 15 ml. of saturated picric acid solution and 1 ml. of saturated sodium hydroxide solution for reading at 520 mμ against a reagent blank.[57]

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49 Peter Stelgens, Hans Wolff, and Kurt Schreier, *Klin. Wochschr.* **28**, 318 (1950).

50 See Volume II, pages 70-72.

51 Fr. Franzen and H. Göden, *Z. ges. exptl. Med.* **116**, 1-12 (1950).

52 N. O. Abdon and Erik Jacobsen, *Skand. Arch. Physiol.* **77**, 100-6 (1937); Charles F. Schaffer, *J. Am. Chem. Soc.* **60**, 2001-2 (1938).

53 André Riegert, *Compt. rend. soc. biol.* **132**, 535-7 (1939).

54 Eugenio E. Vonesch, *Rev. asoc. bioqím. argentina* **13**, 197-210 (1946).

55 J. A. Barclay and R. A. Kenney, *Biochem. J.* **41**, 586-9 (1947).

56 John H. Peters, *J. Biol. Chem.* **146**, 179-86 (1942).

57 E. C. Noyons, *Nederland. Tijdschr. Geneeskunde* **83**, II, 2595-2601 (1939); Cf. J. R. Coffman and F. C. Koch, *J. Biol. Chem.* **135**, 519-30 (1940); Leland C. Clark, Jr., and Haskell L. Thompson, *Anal. Chem.* **21**, 1218-21 (1949).

If the urine is highly pigmented, add to 50 ml. of urine 3 ml. of saturated copper sulfate solution and mix. Add 2 ml. of saturated potassium ferrocyanide solution and mix. Filter after a few minutes. Pass hydrogen sulfide through the filtrate to precipitate copper sulfide and filter. Pass air through the filtrate to drive off excess hydrogen sulfide and dilute to a known volume for development as described for normal urine, starting at "Mix 1 ml. of urine . . . ."

Rat urine. To determine the small amount of creatinine in rat urine mix 5 ml. with 2.5 ml. of saturated picric acid solution and 1 ml. of 10 per cent sodium hydroxide solution. After 10 minutes, dilute to 25 ml. and read against a reagent blank.

Milk. Dilute 10 ml. of milk with a 1.2 per cent picric acid solution to 50 ml. and shake. Add about 1 gram of dry picric acid and shake for 5 minutes. Centrifuge to separate protein material and pour the supernatant fluid through a filter. A substantially protein-free filtrate is obtained for reading against a reagent blank.

Blood.[58] Add 4 ml. of plasma or serum dropwise to 12 ml. of saturated picric acid solution. Warm 10-15 seconds in boiling water and filter at once. To 10 ml. of clear filtrate add 0.5 ml. of 10 per cent sodium hydroxide solution and, if any turbidity develops, filter after 15 minutes. Read at 520 mμ against a reagent blank 20 minutes after adding the alkali.

Tissue. Grind 10 grams of fresh minced tissue with 20 grams of sand to a uniform paste. Add gradually, saturated picric acid solution to give 50 ml. of total solution. Add 1 gram of solid picric acid. Continue rubbing and stirring for 5-10 minutes. The creatinine is extracted and proteins are converted to insoluble picrates. Filter. To 20 ml. of filtrate add 1 ml. of 10 per cent sodium hydroxide solution. Let stand 10 minutes, filter or centrifuge, and read against a reagent blank. Some tissues such as liver and brain do not yield clear filtrates. For these add 2 ml. of a 40 per cent solution of formalin to 10 grams of sample and let stand for 10 minutes before extracting with the picric acid solution.

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[58] Hans Popper, Emil Mandel and Helene Mayer, *Biochem. Z.* 291, 354-67 (1937); Aaron Arkin, Hans Popper and Fred A. Goldberg, *Ann. Internal. Med.* 15, 700-7 (1941); Cf. Roy W. Bonsnes and Hertha H. Taussky, *J. Biol. Chem.* 158, 581-91 (1945); Gyorgy Neumann and Edwin Tower, *Orvosi Hetilap.* 90, 50-3 (1949).

Meat extracts.[59] Dissolve 2 grams of solid or soft meat extract, 5 grams of liquid meat extract, or 10 grams of bouillon cube, in water. Dilute to 50 ml. and filter. Place 2.5 ml. of the filtrate in a porcelain dish with 2.5 ml. of 1:2.5 hydrochloric acid, a few granules of lead, and a little lead powder. Evaporate to 0.5 ml. over a low flame. Dissolve the residue in water and filter. Add 1.5 ml. of 10 per cent sodium hydroxide solution and 20 ml. of saturated picric acid solution. Allow to stand for 5 minutes; then dilute to 100 ml. with water and read at 520 mμ against a reagent blank.

Alternatively,[60] add 15 ml. of freshly prepared 20 per cent trichloroacetic acid solution to 10-20 ml. of 10 per cent solution of meat extract. Shake, dilute to 100 ml., and filter. To 10 ml. of filtrate add 2 ml. of 1:10 hydrochloric acid and autoclave at 130° for 25 minutes. Cool, add 1 gram of acid-washed bleaching earth, centrifuge, and decant. Treat the residue with 10 ml. of 1:1000 hydrochloric acid solution in the same manner. Elute 3 times with 10-ml. portions of a solution containing 1 per cent of sodium hydroxide and 2 per cent of sodium chloride. Centrifuge and decant after each shaking. Neutralize the combined decanted solutions with dilute hydrochloric acid and then dilute to 100 ml. and filter. To 10 ml. of filtrate add 10 ml. of saturated picric acid solution and 1 ml. of 10 per cent sodium hydroxide solution. Allow to stand for 20 minutes, dilute to 100 ml. and read at 520 mμ or 530 mμ against a reagent blank.[61]

By picric acid. Creatine. Urine. As a reagent, add 1 volume of 10 per cent sodium hydroxide solution to 5 parts of picric acid solution which contains 11.75 grams of picric acid per liter.[62] Add 40 ml. of 1:300 sulfuric acid solution to 5 ml. of urine, shake, and filter if a precipitate forms. Autoclave 8 ml. of protein-free sample for 20 minutes at 115-120° and 15 pounds pressure. Allow to cool and add 4 ml. of the alkaline picrate reagent. After 20 minutes, read as the sum of

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59 Lucio Zanni, *Ind. ital. conserve aliment.* **12**, 21-3 (1937); J. Nijs and J. deWolf, *Natuurw. Tijdschr.* **23**, 110-12 (1941); Cf. Baier and Walter, *Z. Untersuch. Lebensm.* **74**, 281-3 (1937); S. Rauschning, *Ibid.* **84**, 26-30 (1942).

60 G. Lagrange, *Bull. soc. chim. Belg.* **51**, 113-29 (1942); Cf. Karel Kacl and Frant. Fink, *Chem. Obzor* **12**, 66-70, 89-92, 101-8 (1937); Karl Wodich, *Österr. Chem. Ztg.* **41**, 12-13 (1938).

61 Alfred Luszczak, *Abh. Hyg.* **17**, 27-39 (1935).

62 John H. Peters, *J. Biol. Chem.* **146**, 179-86 (1942); Cf. Hans Popper, Emil Mandel and Helene Mayer, *Biochem. Z.* **291**, 354-67 (1937); Leland C. Clark, Jr. and Haskell L. Thompson, *Anal. Chem.* **21**, 1218-21 (1949).

creatine and creatinine at 520 mμ against a reagent blank. Subtract creatinine and multiply by 1.16 to give creatine.

Alternatively, treat as creatinine with the exception of heating for 30 minutes at 120° in an autoclave between additions of sodium picrate buffer and sodium hydroxide. Cool the samples immediately after autoclaving. Read as the sum of creatine and creatinine. Subtract creatinine as separately determined and multiply by 1.16.

Rat urine. Add 0.5 ml. of 1:5 hydrochloric acid to a 5-ml. sample and evaporate to less than 2 ml. Heat at that volume for 2 hours. A funnel in the neck of the flask is a suitable condenser. Dilute to 5 ml., add 0.5 ml. of 8 per cent sodium hydroxide solution, and read as for normal urine. The result is the sum of creatine and creatinine. Subtraction of creatinine gives creatine after multiplication by 1.16.

Milk. Precipitate the proteins as for creatinine. Transfer 10 ml. of filtrate to a flask, cover with tinfoil, and heat in an autoclave at 120° for 20 minutes. Let cool, dilute to 25 ml., and use 20 ml. in the same way as for creatinine in milk. The result is the sum of creatine and creatinine. Subtraction of creatinine gives creatine on multiplication by 1.16.

Blood. Transfer 5 ml. of blood filtrate to a tube, add 1 ml. of 1:10 hydrochloric acid, cover the mouth with tin foil, and autoclave at 130° for 20 minutes. Cool, add 5 ml. of the alkaline picrate solution as prepared for creatinine, and let stand for 10 minutes. Dilute to 25 ml. and read as creatinine. Multiply the increase over a separate creatinine determination by 1.16 to get the creatine content.

Blood serum. Mix 2 ml. of serum with 5 ml. of water and add 3 ml. of a freshly prepared 10 per cent trichloroacetic acid solution, dropwise with stirring. Centrifuge and pour off the clear protein-free supernatant layer. Add 1 drop of a 1 per cent solution of *p*-nitrophenol in ethanol. Neutralize 5 ml. of this solution with 33 per cent sodium hydroxide solution drop by drop until the color becomes yellow, showing that all excess trichloroacetic acid has been neutralized. Add 2.5 ml. of 1:1 hydrochloric acid, giving a solution approximately 1:5 with hydrochloric acid. Seal with a tight stopper and incubate at 60-65° for 24 hours. Creatine is converted to creatinine. Cool and take 5 ml. as sample. Add 33 per cent sodium hydroxide solution until the color of the nitrophenol shows neutrality by changing to yellow. Add 5 ml. of a fresh mixture of 25 ml. of saturated picric acid solution and 10 ml. of 3 per cent sodium hydroxide solution. Shake and read after 30

minutes. Subtract the value for creatinine separately found. Multiply the difference by 1.16 to give the value for creatine.

Whole blood. Hemolyze, remove the proteins, and neutralize as for creatinine. Then treat 5 ml. as for creatine in blood serum through the operation of cyclicizing. Take 5 ml. of this solution and complete as for creatine in serum. The result, creatinine plus creatine, less creatinine gives creatine when multiplied by 1.16.

Tissue.[63] Grind a 10-gram sample of tissue to a paste with 20 grams of sand and a little water. Add 5 ml. of 50 per cent trichloroacetic acid solution and 15 ml. of water gradually while continuing the grinding. Dilute to 50 ml. and after about an hour, filter. To 20 ml. of filtrate add 2 ml. of 35 per cent lead acetate solution and then slowly add 10 per cent sodium hydroxide to bring the pH to 9.8. This process will remove chromogenic substances. Dilute to 30 ml. and filter after 15 minutes. Remove excess lead with a drop of 20 per cent potassium acid phosphate. Filter and to 10 ml. of filtrate add about 1 ml. of 1:5 hydrochloric acid. Autoclave for 45 minutes at 120°. Evaporate to about 8 ml., neutralize with 10 per cent sodium hydroxide, and dilute to 10 ml. Add 5 ml. of saturated picric acid solution and 1 ml. of 10 per cent sodium hydroxide solution. After 8 minutes read at 520 mμ against a reagent blank. The result is creatine and creatinine. The result on subtraction of creatinine as separately determined is creatine when multiplied by 1.16.

By sodium 3,5-dinitrobenzoate.[64] *Creatine and creatinine. Tissue.* Extract a 0.5-gram sample of well-disintegrated tissue with 5 ml. of phosphate buffer for pH 6 (See Vol. 1, page 176). After 30 minutes add 5 ml. of 10 per cent trichloroacetic acid solution in 1:2 hydrochloric acid. Reflux for 90 minutes, cool, and filter. Dilute to 25 ml. with 1:35 hydrochloric acid. Reflux 5-10 ml. of filtrate for 1.5 hours as before to complete the conversion of creatine to creatinine.

Prepare [65] an approximately 10 per cent solution of sodium 3,5-dinitrobenzoate by adding slowly and with stirring 20 ml. of 2 per cent sodium hydroxide solution to 2.2 grams of 3,5-dintrobenzoic acid in 200 ml. of water. Filter when the solution becomes colorless. To 2 ml. of colorless test solution add 0.4 ml. of reagent and 0.4 ml. of 4 per cent sodium hydroxide solution. Read against a reagent blank after 10

63 I. Shin-Ichi Shibuya, *J. Biochem.* (Japan) **25**, 701-21 (1937).

64 M. Jipp and F. Menne, *Biochem. Z.* **320**, 316-21 (1950).

65 Adolf Bolliger, *Med. J. Australia,* **1936**, *II*, 818-21.

minutes in the dark at 670 mμ. The result less creatinine as separately determined when multiplied by 1.16 is creatine.

Creatinine. By 3,5-dinitrobenzoic acid. Urine.[66] As reagent suspend 30 grams of pure, 3,5-dinitrobenzoic acid in 420 ml. of water. Add 80 ml. of 10 per cent sodium carbonate solution. When evolution of carbon dioxide ceases, filter and use the filtrate.

Neutralize 5 ml. of 2:25 dilution of urine with 10 per cent sodium hydroxide solution. Use methyl red as an indicator and dilute to 11 ml. Add 10 ml. of the reagent, then 10 ml. of 20 per cent sodium acetate solution and 1 ml. of 10 per cent sodium hydroxide solution, and mix. After 5 minutes dilute to 50 ml. and read at 670 mμ.

Blood. As reagent suspend 20 grams of pure 3,5-dinitrobenzoic acid in 150 ml. of water and add 50 ml. of 10 per cent sodium carbonate solution. Filter when evolution of carbon dioxide ceases. To 10 ml. of tungstic acid-blood filtrate, prepared by adding the reagents in the reverse order,[67] add 3 ml. of the reagent and 0.5 ml. of 10 per cent sodium hydroxide solution. Mix, let stand for 10 minutes, and read at 670 mμ against a reagent blank.

Creatine. By acetyl benzoyl. Muscle. Rub up 1 gram of muscle with 7 ml. of water and a suitable quantity of quartz sand. Add 1 ml. of saturated uranyl acetate solution to precipitate proteins, mix well, and filter. The uranium in the filtrate is of no importance. Add 1 ml. of filtrate to 1 ml. of 60 per cent potassium hydroxide solution and mix. Add 0.2 ml. of a 1 per cent solution of acetyl benzoyl in ethanol. Heat in boiling water for 30 minutes. Cool in ice water and immediately add 1 ml. of 5 per cent hydroxylamine hydrochloride solution. Dilute to 10 ml. and read within 10 minutes.

Creatine. By α-naphthol and diacetyl. Neutralize a sample containing not over 0.01 mg. of creatine and add 2 ml. of 1 per cent aqueous α-naphthol in 6 per cent sodium hydroxide and 16 per cent sodium carbonate. Mix, dilute to 10 ml., and mix. Read at 530 mμ against a reagent blank.

6-Aminopurine, Adenine

Adenine is estimated by reduction with zinc dust and coupling with N-(1-naphthyl)ethylenediamine dihydrochloride.[68] Accuracy is to ±5

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[66] Hans Röttger, *Biochem. Z.* **319**, 359-69 (1949).

[67] Jerome E. Andes, *Am. J. Clin. Path.* **8**, Tech. Suppl. 2, 12-19 (1938).

[68] D. L. Woodhouse, *Arch. Biochem.* **25**, 347-9 (1950).

per cent. Adenine is also read in the ultraviolet[69] at 262 mμ and 248 mμ and compared with a curve. Results are 10-20 per cent below theoretical.

Samples—*Nucleic acids and nucleotides.* Reflux a sample with 1:36 sulfuric acid for 1 hour. In that case omit the addition of acid in the procedure. Determine with N-(1-naphthyl)ethylenediamine dihydrochloride.

Mixtures of adenine and guanine. To a sample containing about 4 mg. of mixed purines add 0.1 ml. of 4 per cent sodium hydroxide solution and 0.7 ml. of redistilled ethylene glycol. As a butanol-water mixture, reflux *n*-butanol with zinc dust and strong alkali for 2 hours; then distil and redistil *in vacuo* to remove impurities giving light absorption in the ultraviolet. Mix 865 ml. of this purified butanol with 135 ml. of water. Heat the sample and add 9.8 ml. of the *n*-butanol-water mixture. Prepare starch for a sorption column by extracting with methanol for 24 hours and drying *in vacuo* at 45°. Introduce the sample solution into a 35 × 120 mm. starch column. Take cuts of the effluent at hourly intervals. Pass 120 ml. of solvent through the column, which will take about 12 hours. Read the fractions in the ultraviolet.

Procedure—*By N-(1-naphthyl)ethylenediamine dihydrochloride.* Measure out 25 ml. of sample solution containing 0.1-1 mg. of adenine. Allowing for acidity already present, add 1.5 ml. of 1:1 sulfuric acid. Add 0.01 gram of zinc dust and incubate at 90° for 30 minutes. Cool and filter through cotton. Mix a 2-ml. aliquot with 1 ml. of 1 per cent sodium nitrite solution. Mix and after 10 minutes add 1 ml. of 0.1 per cent ammonium sulfamate. Mix and after 2 minutes add 1 ml. of fresh 0.1 per cent aqueous N-(1-naphthyl)ethylenediamine dihydrochloride. Dilute to 10 ml. and read after 10 minutes at 480 mμ against a reagent blank.

In the ultraviolet. Read the absorption of the effluent against a solvent blank at 262 and 248 mμ. The quotient of the readings is 1.5 for adenine and 0.7 for guanine. Determine by simultaneous equations.

2-Amino-6-hydroxypurine, Iminoxanthine, Guanine

Guanine is determined by the nitroprusside-ferrocyanide-peroxide reagent used for estimation of guanidine. The guanine is oxidized with

[69] Pehr Edman, Einar Hammersten, Bengt Low, and Peter Reichard, *J. Biol. Chem.* **178**, 395-8 (1949).

potassium permanganate to guanidine.[70] Adenine, xanthine, hypoxanthine, inosine, and uric acid do not interfere. Arginine gives a color which fades 50 per cent in an hour. Hypochlorites and sulfides discharge the color. Creatinine reduces the color rapidly. Creatine is reduced to creatinine and so interferes.

As another technic, all purine bases are completely precipitated with ammoniacal copper sulfate in the presence of excess glucose after which the precipitate is dissolved in acid for estimation of guanine,[71] and also of xanthine, with phosphotungstic-phosphomolybdic acid. Uracil and allantoin are not precipitated. Hypoxanthine and adenine give substantially negligible color. Guanine and xanthine give identical color intensities. A method of separation from adenine and reading both in the ultraviolet was given under adenine (page 337).

Sample—*Tissue.* Cut about 20 grams of tissue into small pieces with scissors. Add 20 grams of dry sea sand which has been refluxed with concentrated hydrochloric acid for 24 hours and washed free from chlorides. Grind to a pulp in a mortar. Prepare acidified ethanol by adding 10 ml. of 1:7.5 acetic acid to 1 liter of absolute ethanol. Add 100 ml. of this acid-ethanol, heated to boiling, to the tissue pulp and transfer from the mortar. Clean out the mortar with 100 ml. more of the acid-ethanol. Heat to boiling for 5 minutes, stirring to prevent bumping. This coagulates blood serum proteins without hydrolysis. Let settle for 1 minute and decant the upper layer to a filter, avoiding transfer of the solid matter. Add another 200 ml. of the acid-ethanol and heat for 5 minutes. Decant the liquid through the filter as before. Discard the filtrate.

Dry the residue in an oven and pulverize in a mortar. Add 200 ml. of boiling 1:750 acetic acid to the pulverized residue and heat to boiling for 1 minute. Let settle and decant through the filter used for filtration of the alcoholic extract. Repeat this extraction 4 more times. Add 200 ml. of boiling acid-ethanol and heat to boiling for 1 minute. Decant through the same filter and repeat the operation twice more. The final extract should not give a white cloud when a small amount of it is mixed with an equal volume of water. These extractions remove non-proteins such as creatine. Extract the residue 5 times with 200-ml.

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[70] O. W. Tiegs, *Australian J. Exptl. Biol. Med. Sci.* 1, 93-6 (1924); R. Hedley Marston, *Ibid.* 1, 99-103 (1924).

[71] George H. Hitchings, *J. Biol. Chem.* **139**, 843-54 (1941); J. N. Williams, *Ibid.* **184**, 627-32 (1950).

portions of cold ether to remove fats, phospholipides, etc. Scrape any residue from the filter paper and add to the other solids. Dry in an oven at 100° with the heat turned off as a safety precaution.

Transfer quantitatively to a Kjeldahl flask. Add 200 ml. of 1:10 hydrochloric acid and heat in an oil bath at 120° with the level above that of the liquid in the flask. Insert a cold finger as a condenser in the neck of the flask. Heat directly with a micro burner for 8 hours after the contents of the flask come to a boil. Filter while hot, retaining the bulk of the sand in the flask. Wash the contents of the flask with ten 20-ml portions of 1:10 hydrochloric acid. Dilute the filtrate, which contains all of the guanine, to 400 ml.

Add about 12 grams of acid-washed diatomaceous earth to 300 ml. of the filtrate to sorb humins. Shake well, filter, and wash. The original dark brown of the hydrolyzate will be reduced to a pale yellow. Neutralize the entire filtrate with 10 per cent sodium hydroxide solution. Add 30 ml. of a solution containing 36 grams of trisodium citrate per 100 ml. of water. This will buffer the solution to pH 8.3 and prevent foaming. Add 30 ml. of 30 per cent sodium bisulfite solution and heat to boiling. Add 30 ml. of a 10 per cent copper sulfate solution slowly to the mixture while boiling. Boil for 2 minutes after the copper sulfate is added and cool. Centrifuge, decant the upper layer, and disperse the precipitate in 100 ml. of water. Centrifuge again. Repeat the washing operation twice more.

Add 1:10 hydrochloric acid to disperse the precipitate. Heat in an oil bath at 120° for about 5 minutes. The precipitate dissolves, except for a small residue. Remove from the bath and dilute to about 10 ml. with water. Heat to boiling again and add 1.6 per cent potassium permanganate solution at the rate of about 2 drops per second until a reddish orange color is obtained. Oxidation of guanine is then complete. Note the volume of permanganate used. Evaporate the oxidized solution to dryness.

Prepare an alcoholic alkali the day before use by mixing 10 ml. of 14 per cent potassium hydroxide solution with 100 ml. of absolute ethanol. Only a trace of carbonate should sediment on standing. The solution will become yellow on standing for a longer time. If yellow in 24 hours redistil the alcohol. Add 10 ml. of this alcoholic alkali to the residue. Break up the mass and mix well. Add 2 ml. of 3 per cent hydrogen peroxide with stirring. This converts manganous to manganic compounds and oxidizes unstable organic substances. If not alkaline to

litmus, repeat the addition of alcoholic alkali but add only 1 ml. more of hydrogen peroxide. When effervescence ceases, add 20 ml. of absolute ethanol and let stand for 30 minutes. Filter on a Gooch crucible and wash with 20-ml. portions of absolute ethanol. When the filtrate totals approximately 90 ml., add 10 ml. of absolute ethanol and 1:20 hydrochloric acid until distinctly acid. Evaporate to dryness.

Add 10 ml. of 1:120 hydrochloric acid and dissolve the residue by heating gently. A slight insoluble residue remains. Cool and add 1 ml. of 10 per cent sodium hydroxide solution. If more than a trace of manganese dioxide now remains as a residue, the estimation is faulty and must be repeated. Freshly precipitated manganese dioxide sorbs guanidine from aqueous alkaline solution. Causes of trouble would be insufficient hydrogen peroxide, insufficient time of standing of the residue with alcoholic alkali, or insufficient stirring and breaking up of the residue in alcoholic alkali.

Filter the alkaline solution of oxidation products through a sintered glass micro filter and rinse with water until nearly 20 ml. of filtrate are collected. This entire sample is developed with nitroprusside-ferrocyanide.

For development with phosphotungstic-phosphomolybdic acid, extract minced tissue with 10 volumes of 5 per cent trichloroacetic acid solution. After 4 hours at 5° filter and heat 10 volumes of filtrate with 1 volume of 1:8 sulfuric acid for 2 hours in boiling water. Neutralize to phenolphthalein with 30 per cent sodium hydroxide solution and make just acid with 1:8 sulfuric acid. Add, for each gram of tissue represented by a 10-ml. aliquot, 0.15 ml. of saturated sodium bisulfite solution and 0.1 ml. of 10 per cent solution of copper sulfate pentahydrate. Heat in boiling water for 2-3 minutes to coagulate and centrifuge. Add minimal additional amounts of reagents if essential. Complete as for urine from "Centrifuge to throw down the precipitate."

Urine. Neutralize a 4-ml. sample and mix with 1 ml. of 10 per cent glucose solution and 4 ml. of 1 per cent copper sulfate pentahydrate in 1:19 ammonium hydroxide. Heat in boiling water for 10 minutes and cool. Centrifuge to throw down the precipitate. Wash the precipitate with water until washings are no longer blue. Add 1 ml. of 1:1 hydrochloric acid to the precipitate and dilute to 5 ml. with water. Heat in boiling water for 5 minutes to dissolve the precipitate. Some sediment often remains. Add 0.5 ml. of 1 per cent potassium ferricyanide solution, mix, and centrifuge for 10 minutes. This precipitates the copper. Develop with phosphotungstic phosphomolybdic acid.

Procedure—*By nitroprusside-ferrocyanide reagent.* Mix 5 ml. of 10 per cent sodium nitroprusside solution, 5 ml. of 10 per cent potassium ferricyanide solution, and 5 ml. of 10 per cent sodium hydroxide solution as reagent. Dilute to 60 ml. and let stand. The mixture is at first very dark but fades to a light yellow in about 30 minutes and is then ready for use. Mix 5 ml. of the reagent with the sample and after 30 minutes dilute to 25 ml. Read against a reagent blank.

By phosphotungstic-phosphomolybdic acid. Mix 2 ml. of the clear sample solution with 1 ml. of phosphotungstic-phosphomolybdic acid reagent (Vol. III, page 116) and add 5 ml. of saturated sodium carbonate solution. The color develops at once and is stable for hours. Read at 660 mμ.

2,6-Dioxopurine, Xanthine

The reaction with phosphotungstic-phosphomolybdic acid is identical with that described for guanine. The method of isolation given there also applies.

4-Ethoxyphenylurea, *p*-Phenetylcarbamide, Dulcin

After acid hydrolysis of dulcin to *p*-phenetidine, this is diazotized and coupled with phenol in alkaline solution to give a reddish-yellow azo dyestuff.[72] Sulfuric acid of 1:12 dilution is satisfactory, but if 1:1 sulfuric acid is used, *p*-phenetidine is not even detectable in the final distillate.[73] Saccharin does not interfere. Sugar is converted to furfuraldehyde and either prevents color development or causes it to be fugitive. Interfering primary amines will ordinarily alter the color. Oxidation with silver nitrate, finally catalyzed with manganese dioxide, is an alternative.[74]

Sample—*General.* Dissolve in 10 ml. or more of water a sample containing not over 0.1 gram of dulcin. If essential oils are present, extract them with two 10-ml. portions of petroleum ether. Wash the combined petroleum ether extracts with 10 ml. of water and add to the sample solution. If sugar is present, saturate the sample solution with salt and extract with four 30-ml. portions of ethyl acetate. Combine the ethyl acetate extracts in a flask, to be subsequently used for hydrolysis

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72 J. Longwell and C. S. Bass, *Analyst* **67**, 14-15 (1942).

73 J. F. Hirst, F. Holmes, and G. W. G. Maclennan, *Analyst* **66**, 450-1 (1941).

74 Andrea Gandini and Silvestro Borghero, *Igiene moderna* **41**, 11-15 (1948).

under the procedure, and distil the ethyl acetate. Dry in a current of hot air. Take up the residue in water and dilute to about 1 mg. of dulcin per ml. for the use of aliquots.

Foods. Digest a sample to contain 10-50 mg. of dulcin in warm water until extraction is complete. Filter and wash with warm water. Add excess neutral lead acetate to defecate, filter, and wash the filter. Extract the filtrate with 10, 10, and 5 ml. of ethyl acetate. Evaporate the filtered extract to dryness and take up in 25 ml. of water for development of an aliquot.

Procedure—*As p-phenetidine.* Reflux 10 ml. of a dulcin solution containing approximately 1 mg. per ml. for 3-4 hours with 50 ml. of 1:12 sulfuric acid. Cool, make alkaline with 40 per cent sodium hydroxide solution, and steam-distil into 15 ml. of 1:120 hydrochloric acid until 500 ml. of distillate has been collected. Dilute 15-20 ml. of the distillate to 50 ml. with water in a 100-ml. Nessler tube, parallel to standards of *p*-phenetidine solution. Add 4 ml. of 1:120 hydrochloric acid to each tube and cool in ice to below 10°. Add 1 ml. of 1 per cent sodium nitrite solution to each tube and after 10 minutes add 0.5 ml. of 1 per cent aqueous phenol solution. Next, add 3 ml. of 8 per cent sodium hydroxide solution to each and read in terms of a *p*-phenetidine standard. Convert *p*-phenetidine hydrochloride to dulcin by multiplying by 1.037.

By oxidation. To 5 ml. of sample add 1 ml. of saturated silver nitrate solution. Heat in boiling water for about 12 minutes and add about 0.1 gram of manganese dioxide. After heating for 1 minute longer, cool, and extract with 10 ml. of isobutanol. Separate the extract, centrifuge to separate water, and dry with anhydrous sodium sulfate. Read at 500 mμ against a reagent blank.

CHAPTER 8

COMPOUNDS WITH INORGANIC RADICALS [1]

VARIOUS inorganic materials have already appeared as substituents in organic compounds, sulfur and chlorine particularly. The types concerned in this chapter are the oxidized acids such as phosphates of various types, sulfates, etc. Some miscellaneous types are included. Naturally there are no class reactions.

ACYL PHOSPHATES

Acyl phosphates such as acetyl-, propionyl-, butyryl-, and glyceryl-phosphate react with hydroxylamine to give an hydroxamic acid which develops a purple color with ferric ion.[2] Under the conditions of use, ester acylamines do not react. Hence, as used, the reaction is specific for acid anhydrides. Aldehydes give the reaction only on heating. The lower limit is about 0.008 mg. of phosphorus as acyl phosphate.

Reactions which complex the ferric ion and therefore reduce the color are those with fluoride, phosphate, oxalate, and sulfate. Citrate does not interfere and the others may be removed when present in interfering concentrations by deproteinizing with zinc hydroxide with calcium chloride present. The color developed with acetyl-, propionyl-, butyryl-, and glyceryl-phosphate is molecularly equivalent.

Procedure—Adjust the pH of 0.5 ml. of sample to 5.5-7.5 and add 0.5 ml. of 28 per cent hydroxylamine hydrochloride solution neutralized by premixing with 0.5 ml. of 14 per cent sodium hydroxide solution to give a pH of about 6.4. As buffer for pH 5.4 add 1 ml. of a mixture of 1 part of 1:175 acetic acid and 4 parts of 1.36 per cent sodium acetate trihydrate solution. Dilute to 3 ml. and after 10 minutes the formation of hydroxamic acid is complete.

Add sequentially, with mixing, 1 ml. of 1:3 hydrochloric acid, 1 ml. of 12 per cent trichloroacetic acid, and 1 ml. of 5 per cent ferric chloride hexahydrate in 1:120 hydrochloric acid. Centrifuge to remove the protein precipitate and read the clear upper layer after 5-30 minutes at

[1] See Volume III, Chapter 1, for details of organization, condensation, etc.

[2] Fritz Lipmann and Constance Tuttle, *J. Biol. Chem.*, **159**, 21-8 (1945).

540 mμ against a reagent blank. The color starts to fade after 30 minutes. Succinic anhydride is suitable for preparation of the calibration curve on an 0.8 molecular equivalent basis. Alternatively use acetamide as standard and heat in boiling water for 10 minutes with 2000 molecular equivalents of hydroxylamine to convert to the hydroxamic acid.

Lecithin

Lecithin is determined by the reaction between phosphoric acid and molybdic acid in the presence of a reducing agent.[3] Various reducing agents are applicable such as stannous chloride,[4] hydroquinone,[5] hydrazine sulfate,[6] and others. The phosphatides are extracted with alcohol and ether, changed to orthophosphoric acid, neutralized, and reacted with the molybdic acid reagent in the presence of the reducing agent to give molybdenum blue. Accuracy is to ±3 per cent.

Lecithin is estimated nephelometrically to ±2 per cent by its phosphate content as silver phosphate.[7] Chloride must be absent. The phosphate content of lecithin is also determined nephelometrically by strychnine molybdate. A nitric acid solution of the reagent gives a yellow suspension but with hydrochloric acid the suspension is colorless and remains so indefinitely.[8] This suspension is probably a phosphomolybdic acid complex of strychnine. If the amount of hydrochloric acid is too small, an insoluble strychnine-molybdic acid compound precipitates; and if too large, the phosphate precipitate is redissolved. No great variation in the amount of strychnine is permissible. The strychnine molybdate reagent is more sensitive to phosphoric acid than is silver nitrate and less sensitive to impurities. Lecithin is determined by hydrolysis and estimation of the liberated choline (page 68).

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[3] A. Grigaut, *Compt. rend. soc. biol.* **91**, 1014-7 (1924).

[4] Georges Deniges, *Ibid.* **84**, 875-7 (1921); Emil Truog and A. H. Meyer, *Ind. Eng. Chem., Anal. Ed.* **1**, 136-9 (1929); B. E. Horrall, *Ind. (Purdue) Agr. Expt. Sta. Bull.* **401**, 31 pp. (1935).

[5] Richard D. Bell and Edward A. Doisey, *J. Biol. Chem.* **44**, 55-67 (1920); A. P. Briggs, *Ibid.* **53**, 13-6 (1922); S. Byall and J. A. Ambler, *Ind. Eng. Chem., Anal. Ed.* **3**, 136-7 (1931); R. N. Chopra and A. C. Roy, *Indian J. Med. Research* **24**, 479-86 (1936); N. F. Ivanova, *Lab. Prakt.*, (USSR) **1939**, Sommelband. 60-4.

[6] Mario Mengoli, *Boll. lab. chim. provinciali* (Bologna) **2**, 62-5 (1951).

[7] W. R. Bloor, *J. Biol. Chem.* **22**, 133-44 (1915); *Ibid.* **24**, 447-60 (1916).

[8] I. Pouget and D. Chouchak, *Bull. soc. chem.* **5**, 104-9 (1909); *Ibid.* **9**, 649-50 (1911); Isidor Greenwald, *J. Biol. Chem.* **21**, 29-36 (1915); Philip A. Kober and Grete Egerer, *J. Am. Chem. Soc.* **37**, 2373-81 (1915).

Samples—*Whole blood.* Add 1 ml. of freshly drawn blood as a slow stream of drops into 25 ml. of a mixture of 3 parts of ethanol with 1 part of ether. Keep in motion during the process. Heat to boiling in a water bath, with constant shaking. Cool, dilute to 100 ml. with alcohol-ether, mix, and filter.

Transfer from 10 to 25 ml. of the alcohol-ether extract containing about 1.2 mg. of lecithin, add a few glass beads, and evaporate to dryness in a water bath. Shake frequently until boiling begins. Dry for about 15 minutes to remove traces of alcohol which would interfere with the subsequent oxidation. Add 1 ml. of a mixture of 7 parts of concentrated sulfuric acid and 3 parts of concentrated nitric acid, and ash at 180°.[9] Slowly add 30 per cent hydrogen peroxide dropwise until the mixture clears and continue heating until all water is removed and the mixture will char again. Repeat this process until the solution remains clear. From this point on, the technic differs according to the method of development to be used.

For development as molybdenum blue by varied reagents, dilute to 8 ml. with water. Add a drop of phenolphthalein solution and neutralize with a 40 per cent sodium hydroxide solution. Dilute to 10 ml.

For reading as silver molybdate, add a drop of phenolphthalein solution and neutralize with a 20 per cent sodium hydroxide solution, free from chlorides and containing but little carbonate. Note the amount of alkali used. Add 1:72 sulfuric acid until just acid, cool, and add 0.4 per cent sodium hydroxide solution, drop by drop, until just alkaline. Add 1 ml. of 10 per cent ammonium sulfate solution and 1.5 ml. of 0.4 per cent sodium hydroxide solution, and dilute to 10 ml.

For reading as strychnine molybdate, add a drop of phenolphthalein solution and neutralize with 20 per cent sodium hydroxide solution, noting the amount used. Make just acid with a drop or two of 1:1 hydrochloric acid, cool, and dilute to 25 ml. Use 10 ml. of this as sample.

Fresh plasma or serum. Treat 3 ml. of fresh plasma or serum with an alcohol-ether mixture as given for whole blood and treat the alcohol-ether extract in the same way.

Corpuscles. Determine lecithin in whole blood and in plasma separately. The difference gives the amount in the corpuscles.

Cerebrospinal fluid.[10] Dry 0.5-1 ml. of fluid and extract the residue with 20 ml. of 3:1 ethanol-chloroform. Evaporate the extract to dryness

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[9] J. H. Roe, O. J. Irish, and J. I. Boyd, *J. Biol. Chem.* **67**, 579-84 (1926).
[10] Fritz Roeder, *Z. ges. Neurol. Psychiat.* **166**, 557-67 (1939).

and continue as for blood, starting at "Add 1 ml. of a mixture of . . ."

The use of chloroform instead of ether in extraction mixture will include alcohol-insoluble phospholipides.

Egg content of pastries.[11] Extract 2 grams of dried, powdered, sifted sample for about 6 hours with 20-30 ml. of hot ethanol. Evaporate some of the solution, filter if necessary, and dilute to 20 ml. with ethanol again. Use an aliquot for development as molybdenum blue.

To calculate to terms of eggs used, one egg yields 0.13 gram of phosphoric acid. Apply a correction for the alcohol-soluble phosphate present in the flour and other ingredients.

Urine. Digest 10 ml. of urine with 20 ml. of concentrated sulfuric acid and 10 grams of potassium sulfate, as for a Kjeldahl determination. When the solution is clear, let the melt cool. Dissolve in water and dilute to 500 ml. Roughly neutralize 25 ml. with 1:1 ammonium hydroxide and filter. Dilute to 200 ml. Use 10 ml. as sample for nephelometric development with strychnine molybdate.

Total phosphorus in sugar. Mix 5 grams of sugar in a platinum dish with 0.2 gram of anhydrous sodium carbonate. Carefully char over a free flame and finally place in a muffle furnace until a white ash is obtained. The temperature should at no time be high enough for the ash to fuse. The presence of sodium carbonate prevents volatilization of phosphoric acid.

When the ash has cooled, dissolve in 1 ml. of 1:1 nitric acid and take up with about 10 ml. of water. The acid hastens the conversion of pyrophosphoric acid to orthophosphoric acid which is the only form which gives the reaction. Excess nitric acid causes low results. Filter and dilute the filtrate and washings to about 90 ml. for development as strychnine molybdate.

For inorganic phosphorus in sugar, dissolve 10 grams of sugar in water, dilute to about 90 ml., and use as sample. For organic phosphorus in sugar, subtract the value for inorganic phosphorus in sugar from the total phosphorus in sugar.

Procedure—*As molybdenum blue. By stannous chloride.* As reagent, dissolve 0.1 gram of pure tin foil in 2 ml. of concentrated hydrochloric acid by heating in boiling water with shaking. Dilute the solution to 10 ml. The reducing reagent must be prepared fresh every 4 or 5 days. Dilute the neutralized sample to 50 ml. Mix and add 2 ml.

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[11] P. Stadler, *Z. anal. Chem.* **109**, 168-70 (1937).

of a mixture of equal volumes of 10 per cent ammonium molybdate and concentrated sulfuric acid. Add 0.5 ml. of stannous chloride reagent. Mix, let stand for 2 minutes, and read at 525 mμ against a reagent blank.

By hydroquinone. To the 10-ml. sample add 1 ml. of a solution made by mixing 25 grams of ammonium molybdate in 300 ml. of water, with 75 ml. of concentrated sulfuric acid diluted to 200 ml. with water. Then add 1 ml. of a fresh 20 per cent solution of sodium sulfite in water. To develop the color, add 1 ml. of a solution containing 0.5 gram of hydroquinone in 100 ml. of water, which contains 1 drop of concentrated sulfuric acid to retard oxidation. Dilute to 100 ml. Heat for 10 minutes at boiling and read at 525 mμ against a reagent blank.

Nephelometrically as silver phosphate. Place 10 ml. of a 1.5 per cent neutralized solution of silver nitrate in each of two 25-ml. glass-stoppered volumetric flasks. Add 10 ml. of prepared sample and an appropriate phosphate standard through funnels having the stems drawn out so that the 10 ml. are delivered in about 15 seconds. Gently rotate during the addition. Rinse any residues in, dilute the contents of each flask to volume, and compare in a nephelometer. Chlorides must be rigidly excluded.

Nephelometrically as strychnine molybdate. As strychnine sulfate solution, use a 2 per cent aqueous solution. As acid reagent, dilute 50 ml. of concentrated hydrochloric acid to 100 ml. For standardization dilute 5 ml. of this to 100 ml. and titrate against 0.5 N sodium carbonate solution. It should require 24 ml. of this diluted 1:1 acid to titrate 30 ml. of 0.5 N sodium carbonate solution.

As strychnine-molybdate solution, add 10 ml. of the standardized 1:1 hydrochloric acid to 2.5 ml. of water. Dissolve 1.5 grams of pure sodium molybdate in this with shaking. Add 1 ml. of strychnine sulfate solution with shaking and let stand overnight. Filter through a hardened or quantitative filter paper.

To each of two 50-ml. flasks add 25 ml. of water and 5 ml. of 1:1 hydrochloric acid. Add 5 ml. of strychnine-molybdate reagent to each. Add 10 ml. of the sample solution to one flask, and 10 ml. of a standard solution to the other, rotating the flasks during the addition. Dilute to volume, mix, and compare in a nephelometer after 3 minutes.

Glycerophosphates

The α- and β- forms of glycerophosphate are determined by difference methods. Inorganic phosphate is first determined. Then α-glycero-

phosphate is converted to glycolic aldehyde phosphate by reaction with periodate, and excess periodate and iodate formed are destroyed with sodium sulfite. The glycolic aldehyde phosphate is then hydrolyzed with hot acid and the resulting orthophosphate measured to give the α-glycerophosphate by difference. Total α- and β- forms is measured on another portion of sample by converting all the glycerophosphate to the α- form and then to orthophosphate. Thereafter β-glycerophosphate is obtained by difference.[12]

The blue color from reaction of naphthoresorcinol with glyceric acid is used for estimation of total glycerophosphates after isolation and saponification.[13] The sample of glyceric acid must be dried before addition of the reagent or a green tinge will develop.

Sample—*Egg lecithin.* Reflux a sample containing about 50 mg. of lecithin for 1 hour with 10 ml. of 0.4 per cent sodium hydroxide in ethanol. Cool, make acid with 1:350 hydrochloric acid, and add 20 ml. of water. Extract with 20, 20, and 20 ml. of 1:19 chloroform-petroleum ether. Dilute the aqueous layer to 100 ml. and use aliquots for determination of inorganic phosphate, and α- and β-glycerophosphate.

Blood. Deproteinize by addition of 5 ml. of 20 per cent trichloroacetic acid solution. Then add 15 ml. of water. When coagulation is complete, filter and use 15 ml. Add 2 drops of methyl orange indicator solution and titrate almost to an orange color with 30 per cent sodium hydroxide solution, completing with 4 per cent sodium hydroxide solution. Next add 2 ml. of saturated neutral lead acetate solution and 10 ml. of ethanol. Mix and let stand for 24 hours to complete precipitation of lead glycerophosphate. Centrifuge and decant the supernatant liquid. Wash the precipitate with 3 ml. of water and again centrifuge and decant. Suspend the precipitate in 2 ml. of water and pass in hydrogen sulfide through a capillary for 15 minutes. Filter, wash the precipitate three times, and dilute to 10 ml. for the development of aliquots as glyceric acid.

Procedure—*Inorganic phosphate.* To a 5-ml. sample containing 0.01-0.05 mg. of phosphorus as orthophosphate, add 1 ml. of 1:2.6 sulfuric acid. Add 1 ml. of 4 per cent sodium sulfite solution and 1 ml. of 7 per cent sodium molybdate solution. Mix by inversion and add 1 ml. of a 1:200 dilution with water of 40 per cent stannous chloride solution

[12] Chester F. Burmaster, *J. Biol. Chem.* **164**, 233-47 (1946).

[13] S. Rapoport, *Biochem. Z.* **289**, 10, 429-32 (1937).

in concentrated hydrochloric acid. Dilute to 10 ml. with water and mix. After 20 minutes, read at 540 mμ against a reagent blank.

α-Glycerophosphate. Add a 5-ml. sample containing 0.01-0.05 mg. of phosphorus to 1 ml. of 1 per cent aqueous periodic acid and 1 ml. of 1:360 sulfuric acid. After 10 minutes, add 1 ml. of 4 per cent sodium sulfite solution and 1 ml. of 1:2.6 sulfuric acid. Heat in boiling water for 1 hour. Cool in water and dilute to 10 ml. Mix and add 0.5 ml. of 1:2.6 sulfuric acid to a 5-ml. aliquot. Complete as for inorganic phosphorus, starting at "Add 1 ml. of 4 per cent sodium sulfite solution. . . ."

From the result read as inorganic phosphate, subtract the predetermined inorganic phosphate. The difference is phosphate originally present as α-glycerophosphate.

β-Glycerophosphate. Add a 5-ml. sample containing 0.01-0.05 mg. of phosphorus to a mixture of 1 ml. of 1:2.6 sulfuric acid and 1 ml. of 1 per cent periodic acid solution. Immerse in boiling water for about 1 hour, maintaining the volume at 5-7 ml. by addition of hot water. Cool, add 1 ml. of 4 per cent sodium sulfite solution, and dilute to 10 ml. with water. Mix a 5-ml. aliquot with 0.5 ml. of 1:2.6 sulfuric acid. Complete as for inorganic phosphorus, starting at "Add 1 ml. of 4 per cent sodium sulfite solution. . . ."

From the result read as inorganic phosphate, subtract the predetermined inorganic phosphate and α-glycerophosphate as inorganic phosphate. The difference is phosphate originally present as β-glycerophosphate.

Total phosphorus. The total phosphorus is equal to the sum of inorganic phosphate and total glycerophosphate as inorganic phosphate. It is a valuable cross-check. Add a sample containing 0.01-0.05 mg. of phosphorus to 1 ml. of 1:2.6 sulfuric acid containing a few glass beads. Heat gently over a micro burner until the solution turns dark. Cool for 1 minute and add 1 drop of 30 per cent hydrogen peroxide. Heat gently till the solution clears and then increase the flame until fumes of sulfuric anhydride are given off. Cool and dilute to 10-ml. Mix a 5-ml. aliquot with 0.5 ml. of 1:2.6 sulfuric acid and complete as for inorganic phosphorus, starting at "Add 1 ml. of 4 per cent sodium sulfite solution. . . ."

As glyceric acid. Use a 2-ml. aliquot of prepared sample containing 0.015-0.40 mg. of glyceric acid. Add 2 drops of concentrated hydrochloric acid and evaporate to dryness on a water bath. Again add 2 drops of concentrated hydrochloric acid and dry again. Chill the tube in cold water and add 2 ml. of a fresh 0.1 per cent solution of naphthoresorcinol in concentrated sulfuric acid. Heat in boiling water for an

hour. Dilute to 25 ml. with concentrated sulfuric acid. Read at 610 mμ against a reagent blank. An alternative blank is the sample treated as described but with sulfuric acid containing no reagent.

Glucose-6-phosphate

Glucose-6-phosphate is reduced in a system with 2,6-dichlorophenol indophenol triphosphopyridine, and a dihydrocoenzyme, *zwischenferment*.[14]

Procedure—The reagent must be prepared. Suspend 400 grams of dried Canadian ale top yeast in 1200 ml. of water for 10 hours at 35°. Centrifuge and precipitate the *zwischenferment* by dilution of the supernatant layer with water and saturation with carbon dioxide. Dry the precipitate over phosphorus pentoxide to give about 6 grams of dry powder. Prepare a mixture of 2 ml. of 0.015 *M* phosphate buffer for pH 8.3 (Vol. 1, page 176), 0.02 mg. of triphosphopyridine nucleotide, 0.02 mg. of 2,6-dichlorophenol indophenol, and 3 mg. of *zwischenferment*. Adjust the 25°, add a known amount of sample, and measure the rate of decolorization of the dye at 600 mμ.

Phytin Phosphorus

Phytin phosphorus is extracted from plant material with hydrochloric acid, filtered, and precipitated from the filtrate with ferric chloride and thiocyanate as ferric phytate.[15] The turbidity caused by the precipitate is read.[16]

Procedure—Extract an 8-gram sample with 200 ml. of 1:17 hydrochloric acid for 3 hours and filter through double paper. To a 50-ml. aliquot of the filtrate add 10 ml. of 0.3 per cent ammonium thiocyanate solution and 107 ml. of water to get a resultant acidity of 0.6 per cent. Prepare ferric chloride solution by dissolving 1 gram of iron per liter in 1:60 hydrochloric acid and add potassium permanganate solution until a faint temporary pink persists throughout the solution. Add this until the pink color of ferric thiocyanate no longer appears to be fading

[14] Erwin Haas, B. L. Horecker, and T. R. Hogness, *J. Biol. Chem.* 136, 747-74 (1940); Erwin Haas, *Ibid.* 155, 333-5 (1944).

[15] W. Huebner and H. Stadler, *Biochem. Z.* 64, 422 (1914); Robert S. Harris and L. Malcolm Mosher, *Ind. Eng. Chem., Anal. Ed.* 6, 320-1 (1934).

[16] Andrea Gandini and Vincenzo Maione, *Ann. Chim. applicata* 37, 154-62 (1947).

and then add 0.25-0.5 ml. more. Allow to stand until the precipitate flocculates, usually 10-20 minutes. Flocculation can be induced by occasional stirring. Shake and read the turbidity of the precipitate.

Octamethyl Pyrophosphoramide

The insecticide, octamethyl pyrophosphoramide, is determined as molybdenum blue,[17] but is interefered with by inorganic phosphates present. Hydrolysis with mineral acid gives dimethylamine which is converted to water-insoluble cupric dimethyldithiocarbamate which gives a yellow color in organic solvents.[18] Primary and tertiary alkylamines do not interfere and secondary alkylamines are not commonly encountered in biological material.[19] The test substance may be largely in the plant rather than on the surface. Hence maceration or exhaustive extraction is essential. Benzene gives low results as compared with chloroform. Either cork or rubber must be avoided as giving yellow interfering colors.

Sample—*Plant material.* Extract with chloroform for 3 hours in a Soxhlet and dilute the extract to contain about 0.005 mg. of octamethyl pyrophosphoramide per ml. Alternatively macerate the sample with a known volume of chloroform in a blender, filter a known volume of the homogenate, and dilute to about 0.005 mg. of test substance per ml.

Procedure—Mix 10 ml. of sample in chloroform, containing about 0.05 mg. of test substance, with 10 ml. of concentrated hydrochloric acid. Evaporate the chloroform and reflux the sample with the acid for 1 hour. Cool and rinse the condenser with water. Transfer to an all-glass Kjeldahl apparatus. Add a drop of antifoam and 10 ml. of saturated aqueous sodium hydroxide, and distil into 2 ml. of 1:10 hydrochloric acid, with the condenser tip immersed in the acid. Dilute the distillate to 25 ml. Add 5 drops of the saturated sodium hydroxide solution and 1 ml. of 0.05 per cent copper sulfate pentahydrate in concentrated ammonium hydroxide. Add 20 ml., accurately measured, of 0.5 per cent solution of carbon bisulfide in chloroform. Shake for 3 minutes and

[17] W. E. Ripper, R. M. Greenslade, and G. S. Hartley, *Bull. Entom. Research* **40**, (Part 4) 481-501 (1950).

[18] H. C. Dowden, *Biochem. J.* **32**, 455-9 (1938); S. A. Hall, J. William Stohlman, III, and M. S. Schechter, *Anal. Chem.* **23**, 1866-8 (1951).

[19] N. V. Sidgwick, ''Organic Chemistry of Nitrogen,'' p. 21, London, Oxford University Press (1937).

withdraw the chloroform layer. Dry the extract with anhydrous sodium sulfate and read within 30 minutes against a reagent blank. Compare with a standard curve prepared from the test substance or pure dimethylamine hydrochloride. If the color is too intense, dilute with 0.5 per cent carbon bisulfide in chloroform.

o-Tolylphosphate

o-Tolylphosphate is determined in commercial tritolyl phosphate, by isolation of the mixed cresols, followed by condensation with benzaldehyde in acid solution.[20] The *o*-compound gives a dyestuff red in acid, and blue-violet in alkaline solution, while *m*- and *p*-cresols give no colored products. Up to 6 per cent of *o*-tolyl ester is determined in tritolyl phosphate, after hydrolysis and extraction. Three per cent or more of phenol or of 2,6-xylenol will give a positive error, but commercial samples usually contain smaller quantities unlikely to cause interference.

Sample—*Tritolyl phosphate.* Hydrolyze in a soda-glass, flask, not Pyrex, by refluxing 4 grams for 2.5 hours with 25 ml. of Cellosolve potash, prepared by refluxing 12.5 grams of potassium hydroxide in 25 ml. of ethylene glycol monoethyl ether for 5 minutes. Cool the hydrolysis mixture and dilute with 150 ml. of water. Extract successively with two 25-ml. portions of ether to remove impurities and discard the extracts. Acidify by the dropwise addition of 1:1 sulfuric acid, with cooling, and extract the acid solution successively with two 25-ml. portions of ether.

Wash the combined ether extracts containing the cresols twice with 50-ml. portions of water. Dry over anhydrous sodium sulfate and evaporate off the bulk of the ether. Distil off the remaining ether and then collect the fraction of mixed cresols, distilling to the dry point.

Procedure—Weigh in a tared hard-glass test tube 14 drops of mixed cresols. Add 14 drops of redistilled benzaldehyde and mix. Heat at 130° for a half-minute and then add dropwise 5 ml. of 3:1 sulfuric acid, with constant stirring. Heat for a total period of 2.5 minutes. Cool for 20 minutes, dilute with 10 ml. of water, and filter by suction. Break up the residue and wash carefully with at least 100 ml. of cold water to remove occluded sulfuric acid. Wash with 40 ml. of water warmed to

[20] J. Haslam and D. C. M. Squirrel, *Analyst* 77, 71-4 (1952).

50-60°, then with 2 ml. of 1:50 ammonium hydroxide solution, and finally with cold water.

Transfer the crucible and contents to a reflux flask and heat for 10 minutes with 15 ml. of methanol to dissolve the resinous material. Filter and wash with methanol to a volume of 30 ml. To a 20-ml. aliquot add 0.5 ml. of 10 per cent sodium hydroxide solution and 12 ml. of water. Dilute to 50 ml. and mix. Filter and read against a 75 per cent methanol blank after 15 minutes.

Disodium *p*-nitrophenyl Phosphate

The purity of disodium *p*-nitrophenyl phosphate—used as a substrate in determination of phosphatase—can be determined by measuring the absorption of the *p*-nitrophenol liberated on treatment with hydrochloric acid followed by bringing the pH to 9.[21]

Procedure—Dissolve 20 mg. of *p*-nitrophenyl phosphate in 1:15 hydrochloric acid and make up to 100 ml. with this acid. Transfer 50 ml. of this solution to another flask and place in boiling water for 3 hours. Dilute to 100 ml. and mix. Add 1 ml. to 9 ml. of 7.6 per cent sodium tetraborate solution and read at 400 mμ. Dilute 5 ml. of the unheated half of the original solution with 5 ml. of water. Add 1 ml. of this to 9 ml. of 7.6 per cent sodium tetraborate solution and read at 400 mμ. Divide the difference in absorption for the heated and unheated solutions by 0.0332, to find the mg. of disodium *p*-nitrophenyl phosphate in the weighed sample.

Long-chain Alkyl Sulfates

The reaction of an anion-active structure with a cation-active compound to form a much larger molecule is applicable to long-chain aliphatic sulfates by selective extraction of the reaction product. Chains shorter than dodecyl are not completely extractable. The technic is to couple with a colored cation-active agent for which rosaniline salts are appropriate.[22] The method is not applicable to fatty acids. The reaction is sensitive enough to permit the analysis of solutions as dilute as 1.5 mg. per liter with an accuracy of ±2 per cent. Excess surface-active

[21] O. A. Bessey and Ruth H. Love, *J. Biol. Chem.* **196**, 175-8 (1952).

[22] Fred Karush and Martin Sonenberg, *Anal. Chem.* **22**, 175-7 (1950); Guy R. Wallin, *Ibid.* **22**, 615-17 (1950).

agent disperses it in the aqueous layer in blue form. Whether rosaniline or pararosaniline is the active ingredient is unknown. Alkalies form a brown chloroform-soluble complex. Nitrates in acid solution interfere. Potassium iodide, iodine, potassium bromide, sodium pentachlorophenate, and calcium chloride interfere. Methylene blue gives similar reactions,[23] but sodium sulfate interferes. The reaction with sodium hypochlorite and *o*-tolidine is also applicable.[24]

Due to the insolubility of the compound formed, 1-amino-5-ethoxybenzothiazole can be used for turbidimetric estimation of the long-chain alkyl sufates [25] at levels as low as 10 ppm. Soaps, ammonia, sodium, calcium, barium, magnesium, zinc, aluminum, iron, carbonate, silicate, borate, phosphate, sulfate, sulfite, chloride, nitrate, and acetate do not interfere. Other sulfated and sulfonated materials do interfere.

Sample—*Textiles.* Extract a sample expected to contain about 0.5 gram of test substance, using 100 ml. of hot isopropyl alcohol. Extract the residue with 20 ml. of hot water. Press out as much as possible. Evaporate the alcohol in boiling water, but do not let it go to dryness. Dilute the residue to 100 ml. with water to develop with rosaniline.

Procedure—*By rosaniline.* Prepare a buffer for pH 6.1 by mixing 5 ml. of 0.3414 per cent monopotassium phosphate and 10.57 ml. of 0.10 per cent sodium hydroxide solution and dilute to 200 ml. As reagent dissolve 15 mg. of rosaniline hydrochloride in 100 ml. of this buffer. Extract several times with 1:1 chloroform-ethyl acetate until the intensity of color in the organic phase remains unchanged on successive extractions.

Mix 1 ml. of this reagent with 100 ml. of sample and adjust to a volume of 5 ml. with the buffer. Extract this mixture with 5 ml. of a 1:1 mixture of chloroform and ethyl acetate. Shake manually about 50 times. Centrifuge to separate the phases and read against a reagent blank. The calibration curve must have been prepared with the same batch of reagent.

By hypochlorite and o-tolidine. As *o*-tolidine reagent add 1 gram of the compound to 5 ml. of 1:5 hydrochloric acid and grind. Take up with about 200 ml. of water, add 495 ml. of 1:5 hydrochloric acid, and

[23] G. H. Jones, *Ibid.* **21**, 652-7 (1949).

[24] Jay C. Harris, *Ind. Eng. Chem., Anal. Ed.* **15**, 254-6 (1943).

[25] C. J. Pedersen, *Am. Dyestuff Reporter* **24**, 137-8 (1935).

dilute to 1 liter. Mix 40 ml. of water, 1 ml. of 300 ppm. sodium hypochlorite solution, and 2 ml. of the *o*-tolidine reagent. Add a known amount of the alkyl benzene sulfonate solution containing about 0.5 mg. of the agent to produce a color. Dilute to 50 ml. and read at 525 mμ.

Turbidimetrically. As reagent, prepare a saturated aqueous solution of pure 1-amino-5-ethoxybenzothiazole hydrochloride. Make 100 ml. of the sample solution just acid to Congo red paper with concentrated hydrochloric acid. Cool and filter if the liquid becomes cloudy. When the temperature is constant, add 2 ml. of the reagent, shake, and again immerse in water to maintain a uniform temperature. After 5 minutes read the turbidity.

2,4-Dinitronaphthyl-7-sulfonic Acid, Flavianic Acid

Many bases form salts with flavianic acid. When reduced by reaction of acid and an active metal, the solution is colorless. In the presence of oxygen, as by aeration, this is converted [26] to an orange-red which is directly proportional to the amount of flavianic acid present. Extracts of muscle and organs are suitable for use as samples. Because there is uncertainty about the purity of recrystallized flavianic acid, the flavianate of hydroxylamine is preferable as standard.

The procedure calls for development of the color in alkaline solution. That obtained in acid solution is even more desirable but fades about 10 per cent within 1 hour. Addition of alkali after aeration alters the color to a greenish yellow. These changes illustrate the unknown nature of the reaction. Picric acid gives no color under the same conditions. Picrolonic acid gives a violet color.

Procedure—Add 1 ml. of sample to 0.5 gram of aluminium turnings. Add 1 ml. of 1:10 sulfuric acid and stopper loosely. Heat in boiling water for 3-10 minutes, during which the solution will become colorless. Cool and transfer the contents without the residue of the aluminum turnings. Add 2.5 ml. of 10 per cent sodium hydroxide solution and mix. Heat in boiling water and pass a current of air by means of a fine glass capillary for 10 minutes. Cool, remove the capillary, dilute to 25 ml., and read.

As a standard dissolve 0.33 gram of purified hydroxylamine flavianate in water and dilute to 100 ml. Each ml. is equivalent to 3 mg. of flavianic acid. The standard solution is colorless but, when treated in

[26] Wilson D. Langley and Andrea J. Albrecht, *J. Biol. Chem.* **108**, 729-39 (1935).

the same way as the sample, gives the color developed from the flavianic acid.

Chondroitinsulfuric Acid

When chondroitinsulfuric acid is hydrolyzed, the glucosamine in the hydrolyzate is an accurate measure of the original acid present.[27] None of the ingredients of cartilage interfere with the development of the color of glucosamine by *p*-dimethylaminobenzaldehyde with accuracy to ±2 per cent.

Sample—*Cartilage.* Put the minced cartilage in ethanol for 24 hours. Remove, press between clean filter papers, and dry *in vacuo* over calcium chloride. Powder and weigh 5 grams of the dry powder for hydrolysis. Reflux with 10 ml. of 1:10 hydrochloric acid in boiling water. After 30 minutes cool in running water and evaporate the hydrolyzate and washings to dryness in an open dish. When cool, add 1 ml. of 5 per cent sodium methylate to the dry residue and mix well. Cool the dish in ice water and add 0.3 ml. of acetic anhydride, drop by drop. After 1 minute dissolve the mixture in 1 ml. of water.

Procedure—Add 1 ml. of absolute ethanol and 0.5 ml. of 30 per cent potassium hydroxide solution to the sample. Boil over a free flame for exactly 8 seconds and cool at once in ice water. When cold add 3 ml. of a 2 per cent solution of *p*-dimethylaminobenzaldehyde in 1:1 hydrochloric acid. Shake for 5 minutes and read against a reagent blank.

Ethereal Sulfates

To determine ethereal sulfates, precipitate free sulfate[28] and interfering substances with barium chloride and remove excess barium ion by sodium carbonate. Then hydrolyze to liberate the ethereal sulfates. For determination of the sulfate so liberated, the turbidimetric method[29] is preferred. It is accurate to 0.007 mg. of sulfur trioxide.

Procedure—*Urine.* Mix 5 ml. of urine with 1 ml. of 1 per cent barium chloride solution in 1:50 hydrochloric acid. Centrifuge and

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[27] W. Winter, *Biochem. Z.* **246**, 10-28 (1932); Torazo Miyazaki, *J. Biochem.* (*Japan*) **20**, 211-22 (1934).

[28] Ivar Sperber, *J. Biol. Chem.*, **172**, 441-4 (1948).

[29] Joseph F. Treon and W. E. Crutchfield, Jr., *Ind. Eng. Chem., Anal. Ed.* **14**, 119-21 (1942).

decant. Mix the decantate with 1 ml. of 5 per cent sodium carbonate solution and allow to stand for a few minutes. Centrifuge and decant. Add 1 ml. of 1:5 hydrochloric acid to 3 ml. of centrifuge and heat in boiling water for 30 minutes. Cool and add 0.1 ± 0.005 gram of barium chloride. Shake the test tube gently to dissolve and read.

Lignosulfonates

These are determinable by the same color with sulfamic acid as tannins. Follow the procedure for tannic acid and tannins (Vol. III, page 46).

3-Amino-4-hydroxyphenylarsonic Acid

Small amounts of this compound are determined in acetarsol, N-acetyl-4-hydroxy-*m*-arsanilic acid, by formation of an azo dye with β-naphthol.[30] The value obtained is high because of coprecipitation, but this is corrected by use of an arbitrary factor.

Procedure—*Acetarsol.* To 5 ml. of cold water at about 5°, add 0.1 gram of sodium bicarbonate and 0.2 gram of acetarsol. Shake to dissolve and add 5 ml. of 1:120 hydrochloric acid at 5°. Add 2 ml. of a freshly prepared 0.5 per cent solution of sodium nitrite and mix. After 3 minutes add 0.05 gram of sulfamic acid, shake well, and leave in the ice bath for 5 minutes.

Add 10 ml. of a cooled, freshly prepared 5 per cent solution of recrystallized *beta*-naphthol in 8 per cent sodium hydroxide solution, and mix. Leave in the ice-bath for 10 minutes and then place in water at 20° for 5 minutes. Read against a reagent blank. Correct for acetarsol carried down by subtraction of 0.125 for a 2-cm. cell.

Arsphenamine

Arsphenamine is determined by the red color formed by diazotizing and coupling with resorcinol.[31] Alternatively, the yellow color of the nitroso derivative is read at 430 mμ.

Procedure—Chill 3 ml. of sample to below 10° and add 2 drops of concentrated hydrochloric acid and 0.5 ml. of 0.5 per cent aqueous sodium

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[30] C. W. Ballard and E. J. Ballard, *Quart. J. Pharm. Pharmacol.* **21**, 487-98 (1948); C. W. Ballard, *J. Pharm. Pharmacol.* **1**, 224-9 (1949).

[31] H. Hullstrüng and J. Nordmeyer, *Klin. Wochschr.* **17**, 854-5 (1938).

nitrite. After 10 minutes add 1 ml. of 0.5 per cent ammonium sulfamate. Mix well and add 0.5 ml. of 1 per cent aqueous resorcinol solution. Read at 570 mμ against a reagent blank.

Phenarsazine Compounds

On nitration of phenarsazine compounds they give the 2,8-dinitro compound. In strongly alkaline solution this forms the red quinonoid salt.[32]

Procedure—Evaporate a sample containing 1-5 mg. to dryness on a steam bath. Add 2 ml. of concentrated nitric acid and evaporate just to dryness on a steam bath. Cool and dissolve the residue in 10 ml. of 50 per cent aqueous acetone and 1 ml. of 10 per cent sodium potassium tartrate solution. Add 0.4 ml. of 10 per cent sodium hydroxide solution, mix, and read against a reagent blank.

Phenyl Mercuric and Ethyl Mercuric Compounds

Phenyl mercuric and ethyl mercuric compounds in chloroform react with dithizone for reading excess reagent at 620 mμ.[33] The method determines 0.05-0.15 mg. which is stable for 3 hours in an acetic acid-acetate buffer.[34] Inorganic mercury, if present, is extracted with hydrochloric acid. Under the conditions there is no interference by manganese, iron, cobalt, nickel, zinc, silver, stannous, mercuric, lead, or bismuth ions. Much copper and, to a lesser extent, mercurous ion interfere. Pyridyl mercuric acetate and *o*-mercuriphenol give a yellow color. The method is operated at pH 2.5-8.7. Accuracy is ±2 per cent. The color with phenylmercuric compounds shows decomposition in an hour; ethyl mercuric compounds are stable for several hours. The maximum absorption of the dithizone compound is at 497 mμ.[35]

By shaking with a mixture of 4 parts of hydrochloric acid and 1 part of water, the phenyl mercury compound is decomposed to metallic mercury with substantially no effect on the ethyl mercury compound.[36] This permits determination of 0.007 mg. of ethyl mercuric phosphate in the presence of 3.6 mg. of phenyl mercuric acetate, and of 0.019 mg.

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[32] H. Barnes, *Analyst* 72, 241-4 (1947).

[33] V. L. Miller, Dorothy Polley, and C. J. Gould, *Anal. Chem.* 23, 1286-8 (1951).

[34] A. K. Klein, *J. Assoc. Official Agr. Chem.* 33, 594-7 (1950).

[35] Gunnar Gran, *Svensk. Papperstidr.* 53, 234-6 (1950).

[36] Dorothy Polley and V. L. Miller, *Anal. Chem.* 24, 1622-3 (1952).

of phenyl compound in the presence of 10 times that amount of the ethyl compound. Mercury recovered in the residual solution after hydrolysis corresponds within 3 per cent to the phenyl mercury compound present. Over-all accuracy to 3 per cent is attainable unless amounts are under 0.002 mg.

Procedure—*Phenyl and/or ethyl mercuric compound.* Add 1 ml. of dithizone-extracted 20 per cent hydroxylamine hydrochloride solution to 17 ml. of 1:3.5 hydrochloric acid in a separatory funnel. Add 20 ml. of 1:4 hydrochloric acid to a second funnel. To a third add 5 ml. of 8.2 per cent sodium acetate solution adjusted to pH 4.5 with acetic acid and extracted with dithizone. Add 11 ml. of chloroform containing 0.01 mg. of dithizone per ml. to the first funnel. Add 15-20 ml. of water to the third funnel.

Add a sample of 0.5-20 ml. containing 0.05-0.1 mg. of the phenyl mercuric and/or ethyl mercuric compound to the first funnel. Only moderate acidity or alkalinity may be present. Shake for 1 minute, let separate, and drain the chloroform extract into the second funnel. Repeat the shaking and transfer the chloroform to the third funnel. Shake, separate, and read the chloroform layer at 620 mμ against chloroform. Compare with the standard curve of excess dithizone as obtained with the same compound, and interpret as either compound or their sum.

Ethyl mercuric compound, both present. Add an appropriate sample, not over 1.4 ml., to 2 ml. of 4:1 (12 N)hydrochloric acid. Wash it in with 3 ml. more of the acid and shake mechanically but gently for 15 minutes. Add 1 ml. of 20 per cent hydroxylamine hydrochloride solution and 12 ml. of refrigerated water. Mix by inversion and add 10 ml. of chloroform containing 0.01 mg. of dithizone per ml. Shake vigorously for 1 minute and drain the chloroform layer into 20 ml. of 1:5 hydrochloric acid and shake. Drain the chloroform into 15-20 ml. of water and 5 ml. of buffer for pH 4.5. Shake, separate, and read at 620 mμ. Determine as ethyl mercuric phosphate. Calculate the phenyl compound by difference from the preceding method which determines both.

Tetraethyl Lead, Lead Tetraethyl

Tetraethyl lead is isolated from organic matter by steam-distillation or air volatilization.[37] After oxidation this is determined as inorganic

[37] M. S. Bykhovskaya, *Gigiena i Sanit.* 13, No. 10, 25-9 (1948).

lead. The reaction of silver nitrate with tetraethyl lead in gasoline first forms ethyl silver which decomposes to colloidal silver and free radicals. In alcohol the latter is yellow to brown and is read photometrically.[38]

Sample—*Organs.* Grind an appropriate sample containing 0.1-1 mg. of tetraethyl lead, dilute to a pulp, and steam-distil 20 ml. Treat the distillate with excess of saturated bromine water which will oxidize 60-70 per cent of the content. Add 40 ml. of concentrated hydrochloric acid and reflux gently to complete the decomposition. Evaporate the aqueous acid to a dry residue of lead chloride. Add 30 ml. of concentrated nitric acid and evaporate to dryness to oxidize any residual organic material. Repeat if the dry lead salt is not white. Dissolve the residue of lead nitrate in 10 ml. of 1:10 nitric acid and use all or an aliquot for estimation of the lead.

Procedure—*As lead.* Determine inorganic lead as described in Volume II, pages 35-45. Each ml. of tetraethyl lead contains 1.0570 grams of lead.

As colloidal silver. Dilute a portion of the gasoline sample with ethanol to contain 0.1-0.5 ml. of tetraethyl lead per gallon. To 20 ml. of diluted sample add rapidly 100 ml. of saturated solution of silver nitrate in ethanol. Close the container with a glass stopper and shake for a few seconds. Read at 520 mμ at 1, 2, 3, and 5 minutes after dumping in the silver nitrate solution. The reading after 2 minutes is usually used.

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[38] V. A. Smith, W. E. Delaney, W. J. Tancig, and J. C. Bailie, *Anal. Chem.* 22, 1230-1 (1950).

CHAPTER 9

STEROLS [1]

STEROLS are complex secondary alcohols. The principal one determined in this chapter is cholesterol. However, the hormones are largely sterol derivatives and therefore they are included in the next chapter because of the close relationship. Procedures for sterols usually depend on the reaction with acetic anhydride and sulfuric acid in some of its many variations.

CHOLESTEROL

The reaction of cholesterol with acetic anhydride and sulfuric acid [2] to produce a green color is also given by the esters. It follows that the reaction may determine total cholesterol, free cholesterol, and cholesterol esters by application of suitable separation technics. The method has been modified through the years, so that today the literature has many references to modifications of the Liebermann-Burchard reaction. Many of the older methods requiring drying and subsequent extraction have been superseded by liquid extraction procedures with chloroform. Trichloroethylene [3] and isopropanol [4] are also used. Cholesterol may be retained by coagulated protein.

When alcohol-ether or alcohol-acetone are used for extraction, they must be completely evaporated before dissolving the sterol in acetic anhydride or chloroform for color development. Error can arise from oxidation by overheating or retardation of color development by water.

Time and temperature, as well as the concentrations of reagents, affect the color development.[5] Temperature effects are simplified if the

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[1] See Chapter 1, Volume III, for details of organization, condensations, etc.

[2] C. Lieberman, *Ber.* **18**, 1804 (1886); H. Burchard, "*Beitrage zur Kentnis der Cholesterine*" (1899); A. Grigaut, *Compt. rend. soc. biol.* **68**, 791-3, 827-9 (1910); *Ibid.* **71**, 441-2, 513-5 (1913); *Ibid,* **72**, 227-8 (1914); W. R. Bloor, *J. Biol. Chem.* **24**, 227-31 (1916).

[3] Marcel Paget and Georges Pierrart, *Compt. rend. soc. biol.* **125**, 654-7 (1937); *Bull. soc. chim. biol.* **21**, 528-36 (1939).

[4] Jorge Gaséon and Eduardo R. Scheggia, *Rev. facultad cienc. quim.* **12**, 31-6 (1937).

[5] William S. Hoffman, *Proc. Soc. Exptl. Biol. Med.* **43**, 38-42 (1940).

method is one of direct rather than photometric comparison.[6] Offsetting that is the usual greater sensitivity obtainable by reading photometrically.[7] Mild saponification of esters and use of acetic acid for neutralization minimize extraneous color development.[8] Extraneous colors are at a minimum over the range 630-660 mμ.[9] Color in chloroform extracts can be removed with acid-treated silica gel [10] or synthetic zeolite.[11] Average checks are obtained within 2 per cent.[12] The maximum color intensity is not affected by temperature variation over the range 0-30°, but the rate of development is.[13] Indirect light does not affect the color.

In serum the color can be developed directly in the presence of toluene sulfonic acid, the Liebermann-Burchard reagent being produced in the sample.[14] Both free and esterified cholesterol are read.

The precipitation of cholesterol as the digitonide is a convenient method of separation from esters and associated substances, or after saponification from associated substances. It is not necessary to remove excess digitonin.[15] The method is accurate to 3 per cent [16] and is suitable for micro estimation, particularly if special micro apparatus is constructed for the purpose.[17] Either aqueous or alcoholic [18] solutions of digitonin are used. Complete precipitation of the digitonide is obtainable in 6-20 per cent ethanol in the presence of hydrochloric acid. Acid promotes coupling.

A material associated with the ester in unsaponified filtrates leads to high values,[19] thus explaining why the results of digitonide precipi-

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[6] J. C. Laborde, J. Nazzari, and J. Morato Manoro, *Anales asoc. quim. argentina* **33**, 19-29 (1945).

[7] Robert Begg, *Can. J. Med. Tech.* **2**, 137-9 (1940).

[8] Warren M. Sperry and Florence C. Brand, *J. Biol. Chem.* **150**, 315-24 (1943).

[9] Michael Pijoan and Carl W. Walter, *J. Lab. Clin. Med.* **22**, 968-72 (1937).

[10] J. T. Ireland, *Biochem. J.* **35**, 283-93 (1941).

[11] H. Gershberg and J. C. Forbes, *J. Lab. Clin. Med.* **27**, 1439-43 (1942).

[12] David R. Muirhead, *Can. J. Med. Tech.* **2**, 68-9 (1941).

[13] M. Macheboeuf and J. L. Delsal, *Bull. soc. chim. biol.* **24**, 296-309 (1942).

[14] Sidney Pearson, Sidney Stern, and Thomas H. McGavack, *Anal. Chem.*, **25**, 813-14 (1953).

[15] Rudolph Schoenheimer and Warren M. Sperry, *J. Biol. Chem.* **106**, 745-60 (1934); Francis F. Foldes and B. Craig Wilson, *Anal. Chem.* **22**, 1210-13 (1950).

[16] Fred Fitz, *J. Biol. Chem.* **109**, 523-7 (1935).

[17] Daniel J. Cavanaugh and David Glick, *Anal. Chem.* **24**, 1839-41 (1952).

[18] Herbert K. Allen and J. T. Bryant, *J. Franklin Inst.* **229**, 115-25 (1940).

[19] Rachel M. Smith and Alexander Marble, *J. Biol. Chem.* **117**, 673-84 (1937).

tation are lower than by direct extraction.[20] When cholesterol is precipitated as the digitonide, the precipitate can be decomposed for determination as the free sterol.[21] Digitonin itself gives some color, screened out by reading at 610-630 mμ.[22] A green color with anthrone in concentrated sulfuric acid is developed by treatment of the digitonide.[23]

The reaction is also given by coprosterol, various phytosterols, and in modified manner by chortosterol, isocholesterol, oxydimorphine,[24] cholatrienic acid, and other bile derivatives. Applied to cholesterol isolated from egg whites, it is a convenient estimation of the degree of contamination by egg yolk, even to the extent of a fraction of a per cent.[25] The method is applied to determination of cholesterol and 7-dehydrocholesterol in the presence of each other.[26] It depends on a difference in rate of reaction and is applied to skin in which both are present.

Cholesterol is also precipitated as the pyridinium cholesteryl sulfate.[27] This is by treating the cholesterol in carbon tetrachloride with pyridine followed with chlorosulfonic acid. Precipitation is 97 per cent efficient. The precipitate is dissolved in acetic acid and developed with sulfuric acid and acetic anhydride.

Cholesterol can also be precipitated by addition of a zinc salt followed by potassium ferrocyanide.[28] On treatment of the precipitate with sodium hydroxide the cholesterol is extractable. It is advantageous to add egg albumin to acidified urine when sterols are to be precipitated with sodium tungstate.[29] Cholesteryl esters are hydrolyzed by 50 per cent ethanol[30] or 5 per cent sodium ethylate.[31] Evaporation of an

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[20] E. C. Noyons, *Biochem, Z.* **298**, 391-5 (1938).

[21] F. E. Kelsey, *J. Biol. Chem.* **127**, 15-22 (1939); G. Popjak, *Biochem. J.* **37**, 468-70 (1943).

[22] Stuart Sturges and Arthur Knudson, *J. Biol. Chem.* **125**, 543-50 (1938).

[23] Thomas V. Feichtmeir and Julio Bergerman, *Am. J. Clin. Pathol.*, **23**, 599-602 (1953).

[24] Raymond Latarjet and André Husson, *Compt. rend. soc. biol.* **125**, 683-6 (1937).

[25] J. H. Cook and V. C. Mehlenbacher, *Ind. Eng. Chem., Anal. Ed.* **18**, 785-8 (1946).

[26] P. R. Moore and C. A. Baumann, *J. Biol. Chem.* **195**, 615-21 (1952).

[27] Albert E. Sobel and A. Margot Mayer, *Ibid.* **157**, 255-64 (1945); Albert E. Sobel, Jerome Goodman, and Monte Blau, *Anal. Chem.* **23**, 516-19 (1951).

[28] M. Paget and G. Guyader, *Bull. biol. pharm.* **1937**, 176.

[29] Maurice Bruger and Sylvia B. Ehrlich, *J. Lab. Clin. Med.* **27**, 1093-5 (1942).

[30] Miguel Noriega del Aguila, *Bol. soc. quim. Peru* **2**, 217-18 (1936).

[31] E. C. Noyons and M. K. Polans, *Biochem. Z.* **303**, 415-24 (1940); J. L. Delsal, *Bull. soc. clin. biol.* **26**, 239-46 (1944).

ethanol-ether extract containing benzyltrimethylammonium hydroxide results in complete hydrolysis.[32]

When a solution of cholesterol in glacial acetic acid is treated with acetyl chloride and anhydrous zinc chloride, it develops an eosine-red color detectable in a 1:80,000 solution.[33] After warming for a few minutes, this color changes to a very stable golden brown also suitable for colorimetric estimation.[34] The esters of cholesterol as well as ergosterol and phytosterol give the reaction. Cholesterol esters give results 10 per cent low, indicating that saponification is incomplete. Isovaleryl chloride and benzoyl chloride give more intense colors than acetyl chloride. A solution of *o*-nitrobenzoyl chloride gives a lighter color. The color is more distinct and more stable than that with acetic anhydride and sulfuric acid.[35]

A method developed for ergosterol with antimony trichloride and acetyl chloride (page 375) is applicable to cholesterol. As there shown it gives a maximum at 360 mμ at 20-25 minutes with $E^{1\%}_{1\,cm.}$ of 99.9. By use of a 30 per cent solution of antimony trichloride as reagent this is stepped up to 168.8. For the original reagent 0.01-0.1 mg. per ml. is suitable; for the second, 0.006-0.06 mg. per ml.

In the absence of estrone, estradiol, equelin, androsterone, testosterone, and corticosterone the reaction of dimethylaminobenzaldehyde, *m*-dinitrobenzaldehyde, or salicylic aldehyde can be applied to a 0.005-0.25-mg. sample of cholesterol.[36] Procedures for separation as the digitonide and estimation with phosphomolybdic-phosphotungstic acid[37] or nephelometrically[38] have fallen by the wayside.

Cholesterol adds two bromine atoms. When a solution of cholesterol is treated with bromine, the bromine reacts quantitatively with the cholesterol. On addition of Fast Green FCF thereafter, the bromine decolorizes the dye quantitatively. The excess of dye, in the absence of

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[32] Andre C. Kibrick, Thelma Roberts, and Sol Skupp, *Arch. Biochem. Biophys.* 32, 9-13 (1951).

[33] L. Tschugaeff and A. Gasteff, *Ber.* 42, 4631-4 (1909).

[34] August L. Bernoulli, *Helv. Chim. Acta* 15, 274-86 (1932).

[35] A. R. Rose, F. Schattner, and W. G. Exton, *Am. J. Clin. Path., Tech. Suppl.* 5, 19-23 (1941).

[36] Karl Wulfert, *Acta Chem. Scand.* 1, 818-32 (1947).

[37] Shun-ichi Yoshimatsu, *Tohoku J. Exptl. Med.* 17, 147-53 (1931).

[38] O. Muhlbock, C. Kaufmann and H. Wolff, *Biochem. Z.* 246, 229-46 (1932); *Klin. Wochschr.* 11, 284-5 (1932).

other materials reactive with bromine, measures the cholesterol content.[39] Readings are reproducible to ±2 per cent.

In the absence of other reducing substances, sterols are determinable by reduction of dichromate in strong sulfuric acid. Details are given under lipids (Vol. III, page 325).

Samples—*Serum. Total cholesterol.*[40] Transfer 10 ml. of chloroform to a 15 × 150 mm. glass-stoppered test tube, add 0.2 ml. of serum, and stopper tightly. Prepare a blank at the same time by adding 0.2 ml. of distilled water to 10 ml. of chloroform. As standard add 0.2 ml. of distilled water to 8 ml. of chloroform and 2 ml. of a cholesterol standard containing 0.1 mg. per ml. Tubes closed with chloroform-washed corks are less satisfactory. Shake mechanically for 5 minutes, add 1.5 gram of anhydrous magnesium sulfate to each tube, and stopper again. Mix immediately to avoid caking of the reagent added and shake again mechanically for 5 minutes. This removes moisture from the chloroform and precipitates serum proteins. Add 0.5 gram of Fuller's earth and stopper. Mix vigorously for 10 seconds by hand and centrifuge for 10 minutes. Pipet out a suitable aliquot of the supernatant liquid for development of color by acetic anhydride and sulfuric acid.

Another extraction technic [41] is based on heating a mixture of plasma or serum with acetic anhydride-dioxane solution for 30 minutes, to accomplish simultaneously extraction of cholesterol and cholesterol esters, precipitation of proteins, and conversion of water present into acetic acid.

Pipet 0.2 ml. of clear, unhemolyzed serum or plasma into a 15 × 150 mm. test tube containing approximately 5 ml. of 3:2 acetic anhydride-dioxane mixture. Mix by shaking. Heat in boiling water for 30 minutes with occasional shaking. Filter and wash the original tubes with 0.25-ml. portions of acetic anhydride-dioxane solution until the final volume of the filtrate is 5 ml. Develop the color by the technic specified for this solvent mixture.

Alternatively,[42] to 0.2 ml. of serum add 5 ml. of acetic anhydride, shake, and heat in boiling water for 20-30 minutes. The precipitated

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39 H. T. Gordon, *Anal. Chem.* **23**, 1053-8 (1951).

40 George R. Kingsley and Roscoe, R. Schaffert, *J. Biol. Chem.* **180**, 315-328 (1949).

41 Abraham Saifer and O. F. Kammerer, *Ibid.* **164**, 657-77 (1946); Abraham Saifer, *Am. J. Clin. Path.* **21**, 24-32 (1951).

42 A. Sols, *Rev. españ. fisiol.* **3**, 225-41 (1947).

protein adheres to the wall, permitting the clear solution to be decanted off. Cool and use as sample for reading the color after addition of 0.15 ml. of concentrated sulfuric acid.

Esterified cholesterol. Prepare the reagent by adding 0.4 gram of digitonin to 400 ml. of chloroform. Mix for 5 minutes in a blender, cool, and mix again for 5 minutes. Alternatively shake mechanically for 30 minutes and store for 1 week at 37°. Shake the reagent immediately before use. Add 0.2 ml. of serum to 10 ml. of the digitonin reagent. As a blank, similarly add 0.2 ml. of water. Stopper and shake mechanically for 5 minutes. Add 1.5 grams of anhydrous magnesium sulfate and stopper. Mix immediately and shake mechanically for 5 minutes. Add 0.2 ml. of acetic anhydride, stopper, and mix vigorously for 10 seconds. Add 0.5 gram of fuller's earth, and stopper. Mix by hand for 10 seconds, centrifuge for 10 minutes, and use a portion of the supernatant layer in the procedure for development by acetic anhydride and sulfuric acid.

Blood, plasma, or serum.[43] *Total cholesterol.* Prepare about 75 ml. of a mixture of 3 parts of redistilled ethanol and 1 part of ether. Add slowly, from a pipet, 3 ml. of whole blood, plasma, or serum. Shake during the addition to prevent clumping of the precipitated material. Heat to boiling in a water bath, shake constantly, cool to room temperature, dilute to 100 ml. with alcohol-ether mixture, and filter. The extract can be stored in the dark.

For reading as mixed free and esterified cholesterol, carefully evaporate 10 ml. of the extract just to dryness on a water bath. Extract the dry residue with 3-4 ml. of chloroform, evaporate to 1 ml., and decant into a 10-ml. calibrated glass-stoppered cylinder. Repeat this extraction 3 times. Dilute the combined extracts to 5 ml. The colorless solution is not necessarily clear. Use all, or an aliquot, for development with acetic anhydride and sulfuric acid.

For saponification for total cholesterol, evaporate an aliquot of the alcohol-ether extract to a paste on a steam bath. Add to the sample 10 ml. of a solution of 1:1 alcohol-ether and 0.3 ml. of 35 per cent potassium hydroxide solution. Bring the mixture to a boil and then set aside for a half hour at 37°. Neutralize by adding 1 ml. of hydrochloric acid solution so prepared that this is enough to make 0.3 ml. of the potassium hydroxide solution definitely acid to litmus. Evaporate to a paste on

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[43] F. E. Kelsey, *J. Biol. Chem.* 127, 15-22 (1939); Cf. A. P. Kenny, *Biochem. J.*, 52, 611-19 (1952).

the steam bath. Keeping the sample warm, add 10 ml. of petroleum ether and boil on a steam bath until half of the solvent has evaporated. Decant the petroleum ether into a 15-ml. centrifuge tube. Extract the residue four times. Concentrate the combined extracts to 0.5 ml. for development of color with acetic anhydride and sulfuric acid.

Free cholesterol. For determination of free cholesterol on the extract, it must be free of phospholipids because they are precipitated by digitonin. Add 4 ml. of a 0.2 per cent solution of digitonin in ethanol to an aliquot of the sample extract. Evaporate to dryness in a water bath and dissolve contaminating substances by boiling the precipitate with 12 ml. of petroleum ether. Centrifuge for 3-4 minutes. Discard the supernatant liquid and repeat this extraction twice. The decantates contain the cholesterol ester.

Decompose the precipitate by adding 7 ml. of benzene and boiling slowly for 45 minutes. Maintain a volume of more than 5 ml. of benzene by adding more as needed. Concentrate the volume to 3 ml. after the heating and add enough petroleum ether to bring the volume to 12 ml. Stir and digitonin will be precipitated. Centrifuge and remove the supernatant liquid which contains the purified cholesterol. Extract the precipitate 3 times. Combine the decantates and evaporate to dryness on a steam bath. Remove all traces of solvent with a gentle stream of air. Add exactly 5 ml. of chloroform to the sample for development of color with acetic anhydride and sulfuric acid.

Urine.[44] Gently reflux a 500-ml. sample with 50 ml. of concentrated hydrochloric acid and 100 ml. of chloroform for 10 minutes. Cool, shake for 3 minutes, and separate the chloroform layer. Extract with two 100-ml. portions of chloroform. Discard the sample and wash the combined extracts with two 30-ml. portions of 30 per cent sodium hydroxide solution and then with two 30-ml. portions of water. Evaporate the washed extract to dryness on a water bath and take up the residue in acetone. Precipitate the sterol with 2 ml. of 0.4 per cent digitonin solution and separate by centrifuging. Wash with a 2:1 mixture of ether and acetone until the washings are colorless. Take up the dry precipitate in 1 ml. of glacial acetic acid and 5 ml. of chloroform as a sample for development of color with acetic anhydride and sulfuric acid.

Liver.[45] Grind the sample thoroughly with anhydrous sodium sulfate. Prepare a mixture of 15 ml. of anhydrous acetone and 5 ml. of absolute

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[44] W. M. B. Davidson, *Glasgow Med. J.* **30**, 216-21 (1949).

[45] Louise F. Potter, *Science* **102**, 333 (1945).

ethanol. Grind the sample successively with 10, 5, and 5 ml. of the solvent mixture. Shake the residue with 15 ml. of anhydrous ether for 10 minutes. Separate the ether layer and evaporate to dryness *in vacuo* protected by nitrogen. Take up the residue in a known volume of chloroform and use an aliquot for development of color with acetic anhydride and sulfuric acid.

Skin tissue.[46] Remove hair with a razor and as much as possible of the fatty layer. Dry in a slow current of air at 32-36°. Cut into small pieces, dry further, and weigh. Reflux about 50 mg. gently with 3-4 ml. of a 1:1 absolute acetone-ethanol mixture for 3 hours. When cool dilute the contents and rinsings of the reflux to 10 ml. with the solvent mixture. Filter if cloudy.

Free cholesterol. To an aliquot of 2 ml. add 3 drops of 1:5 hydrochloric acid and 1 ml. of 0.3 per cent digitonin in 90 per cent ethanol. Place in a glass jar, cover, and let stand. Reasonable accuracy is obtainable in an hour, but 12-hours' standing is preferable. Centrifuge and remove the upper layer which contains the esters. Stir the precipitate with about 2 ml. of 1:2 anhydrous acetone-ether. Centrifuge, decant, and wash twice more. Dry the precipitate and proceed as under free cholesterol in blood, plasma, or serum, starting at "Decompose the precipitate by adding 7 ml. of benzene. . . ."

Total cholesterol. Saponify a 1-ml. aliquot of the extract with a drop of 30 per cent potassium hydroxide at 37° for 2 hours. Cool and neutralize to phenolphthalein with 1:5 hydrochloric acid. Add water to dissolve the salt, then 1 ml. of 0.3 per cent digitonin in 90 per cent ethanol. Complete as for free cholesterol, starting at "Place in a glass jar, cover, and let stand."

Rat skin. Both cholesterol and 7-dehydrocholesterol. Unhair with sodium sulfide and wash with water. Stretch on pins and dry at 37° overnight. Complete for 4 hours at 60° *in vacuo.* Cut into fine pieces and extract with 1:1 absolute acetone-ethanol in a Soxhlet for 24-36 hours. Filter and dilute with the solvent to a known volume such as 10 ml.

Free sterols. Dilute an aliquot of the extract containing 0.125-0.5 mg. to 3 ml. with 1:1 absolute acetone-ethanol. Add 1 ml. of 0.5 per cent digitonin in 80 per cent ethanol. After 12 hours at room temperature, centrifuge and complete as for free cholesterol in blood, plasma, or serum (page 367) from "Evaporate to dryness . . ." to

[46] Stuart Sturges and Arthur Knudson, *J. Biol. Chem.* 125, 543-50 (1938).

"... stream of air." Use as sample for both cholesterol and 7-dehydrocholesterol in the presence of each other.

Total sterol. Incubate 3 ml. of solution and 0.1 ml. of 7.5 per cent potassium hydroxide solution at 37° for 2.5 hours. Neutralize with 1:1 hydrochloric acid and carry through the steps for free sterols (page 366) from "Evaporate to a paste. . . ."

Organic tissue. Remove the fat, shred finely, and weigh a 300-mg. sample. Cover the tissue with absolute ethanol and after a half hour place in a 100° oven for an hour. Keep under vacuum overnight, weigh, and add 3-4 ml. of 1:1 absolute acetone-ethanol mixture. Reflux gently for 3 hours. Continue as for skin tissue from "When cool dilute the contents . . . ," applying the methods for free cholesterol and total cholesterol.

Liver oils.[47] Boil 0.5 ml. of liver oil with 20 ml. of 0.9 per cent sodium chloride solution for 30 minutes. Agitate from time to time to facilitate driving off the volatile fatty acids. Add 20 ml. of 25 per cent potassium-hydroxide solution and heat in an autoclave at 150° for 30 minutes. When cool, proceed as for skin tissue, starting at "*Free cholesterol.* To an aliquot of 2 ml. . . ."

Wool Wax.[48] Dissolve 200 mg. in 1:4 ethanol-dioxane and dilute to 100 ml. Mix 5 ml. with 0.1 gram of 50 per cent potassium hydroxide solution and a drop of 1 per cent alcoholic phenolphthalein. Boil gently for 1 minute, stopper loosely, and incubate at 50-60° for at least 3 hours. Cool and neutralize by dropwise addition of 1:2 hydrochloric acid. Add 2 ml. of a 1 per cent solution of digitonin in 80 per cent ethanol and boil gently. Add about 6 ml. of water and boil for 2-3 minutes. Cool and hold at 20° for at least 2 hours, preferably overnight.

Centrifuge to throw down the digitonides and asiprate off the supernatant liquid. Wash the digitonides with 5 ml. of 1:1 acetone-ether and then twice with 5-ml. portions of ether. Remove the last traces of ether at 50°, preferably with a vacuum. Dissolve the precipitate in 12.5 ml. of glacial acetic acid and make up to 100 ml. with chloroform. Develop with acetic anhydride and sulfuric acid.

Skim-milk powder.[49] Transfer 2 grams of skim-milk powder to an extraction flask. Mix 10 ml. of water and 2 ml. of concentrated am-

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[47] K. Hotta and M. Kawaji, *Nagoya J. Med. Sci.* **9**, 23-4 (1935).

[48] Francis E. Luddy, Arthur Turner, Jr., and John T. Scanlan, *Anal. Chem.*, **25**, 1497-9 (1953); cf. Heinz Duewell, *Ibid.* **25**, 1548-50 (1953).

[49] Lincoln M. Lampert, *Ind. Eng. Chem., Anal. Ed.* **2**, 159-62 (1930).

monium hydroxide with the sample. Add 10 ml. of ethanol and shake for one-half minute. Add 25 ml. of ether and shake for one-half minute. Add 25 ml. of petroleum ether and shake for one-half minute. If an emulsion is formed, add a few ml. of ethanol to break it. Centrifuge or let stand a few minutes and pour off the clear layer. Repeat the extraction twice more, using only 5 ml. of ethanol. Combine the extracts and evaporate until the residual liquid becomes cloudy due to insolubility of fat in the residual ethanol.

To the residue add 15 ml. of ethanol and bring to a boil.[59] Add 1 ml. of a 50 per cent solution of potassium hydroxide in water. Cover with a watch glass and boil for 10 minutes to saponify the fat. Let cool and wash down thoroughly with 30 ml. of ether. Transfer the ether and water layers to a separatory funnel with 30 ml. of ether. Then further wash the saponified material in with two 25-ml. portions of water. Rotate the funnel but do not shake, so as to avoid persistent emulsions. When such emulsions are formed, break them with ethanol. The ether separates readily from the aqueous layer. Withdraw the soap solution. Wash the ether layer with 50-ml. portions of water until the washings are neutral. Evaporate the ether layer to dryness. Dissolve the clear yellow oil in chloroform and dilute to 50 ml. with chloroform. It should be clear, but it may be colored yellow by carotinoids. Use an aliquot for estimation by acetic anhydride and sulfuric acid.

Butter. Use 0.5 gram, following the detailed method for skim-milk powder.

Egg yolk. Use 0.3 gram of dry yolk or 1 gram of fresh or frozen yolk, following the detailed method for skim-milk powder.

Egg white. Swirl a 10-gram sample with 25 ml. of concentrated ammonium hydroxide, add 10 ml. of ethanol, and shake for 1 minute. Add 25 ml. of ether and shake for 1 minute. Add 25 ml. of petroleum ether boiling at 35-38° and again shake for 1 minute. If an emulsion does not break on standing, add 5 ml. of ethanol. Decant the clear solvent layer. Add 5 ml. of ethanol and shake for 1 minute. Further extract, starting at "Add 25 ml. of ether . . ." above. Repeat this extraction once more, making three extractions combined. Evaporate the extracts on a steam bath until only a few ml. of ethanol remain. Cool, add 15 ml. of ethanol, and bring to a boil. Add 1 ml. of 50 per cent aqueous potassium hydroxide and boil gently for 10 minutes. Cool and rinse

[59] Raymond Hertwig and L. H. Bailey, *J. Assoc. Official Agr. Chem.* 9, 122-4 (1926).

down the sides with 30 ml. of ether. Transfer to a separatory funnel rinsing in with two 25-ml. portions of water. Rotate the funnel but do not shake. Break any emulsion with 5 ml. of ethanol. Withdraw the aqueous soap solution and wash the solvent with two 50-ml. portions of water. If the second washing is alkaline to phenolphthalein, wash successively with additional portions of water until the washings are neutral to phenolphthalein. Evaporate the ether solution until no moisture remains and take up in 5 ml. of chloroform for development with acetic anhydride and sulfuric acid.

Pastes. Extract a suitable sample of well mixed and dried paste in Soxhlet extractor with ether for 6 hours. Evaporate the ether extract to dryness and dissolve in chloroform. Filter, if necessary, dilute to a known volume, and use an aliquot for development with acetic anhydride and sulfuric acid.

Ice cream. Select a sample according to the expected egg content and proceed as for skim-milk powder.

Procedure—*By acetic anhydride and sulfuric acid. Chloroform extract.* Prepare a fresh reagent by cautiously mixing in an ice bath, 4 parts of acetic anhydride and 1 part of concentrated sulfuric acid, each precooled to 0°.

Transfer an aliquot of chloroform solution containing about 1 mg. of cholesterol to a cuvet and add 2 ml. of the freshly prepared reagent. Mix by twirling—do not invert. Read at 625 mμ against a chloroform blank. Repeat the readings at about 5-minute intervals at 25° or 10-minute intervals at 20° until a maximum color has been developed.

Esterified cholesterol produces about 125 per cent of the color produced by free cholesterol. Therefore the results calculated according to the colorimetric readings are too high by 20 per cent of the apparent esterified cholesterol.

Let

$$A = \text{apparent total cholesterol}$$
$$B = \text{apparent esterified cholesterol}$$

and

$$A - 0.20B = \text{true total cholesterol}$$
$$B - 0.20B = \text{true esterified cholesterol}$$

Free cholesterol is the difference between these two values.

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[51] Arthur E. Teeri, *J. Biol. Chem.* **156**, 279-281 (1944).

[52] Samples for development of the method were calf blood.

Cholesterol esters by direct reading.[51, 52] Dry an aliquot of the cholesterol-cholesterol ester extract, usually in alcohol-ether. Transfer to a 15-ml. centrifuge tube with three 3-ml. portions of petroleum ether. Add 0.1 ml. of 0.3 per cent digitonin in 90 per cent ethanol. Invert occasionally over a 10-minute period to mix. Centrifuge and decant the supernatant liquid through a cotton plug. The solution contains the cholesterol esters. Evaporate to dryness in a small beaker. Extract with chloroform and determine cholesterol by the method applied to the same extract for total cholesterol.

Directly on serum. Add successively to a tube 0.1 ml. of serum, a solution containing 12 grams of 0.1 ml. of glacial acetic acid, 0.5 ml. of *p*-toluenesulfonic acid per 100 ml. of glacial acetic acid, and 1.5 ml. of acetic anhydride. Let stand without mixing until cooled to room temperature and add 0.2 ml. of concentrated sulfuric acid. Mix at once, taking care that the precipitate is completely dissolved. Let stand for 20 minutes and read at 550 mμ against a reagent blank.

By ferric chloride.[53] *Serum.* As reagent add 1 ml. of 10 per cent ferric chloride in acetic acid to concentrated sulfuric acid and dilute to 100 ml. with the acid. Dissolve 0.1 ml. of serum in 3 ml. of glacial acetic acid and add 2 ml. of the reagent. Color develops in 1 minute and is stable for hours. Read at 560 mμ as the total of free and esterified cholesterol.

By acetic acid-dioxan solution. The volume of sample solution should be 5 ml. Add 0.25 ml. of concentrated sulfuric acid from a micro-buret. Mix the contents of the tube and let it stand at room temperature. At the end of the 5-minute interval, place the tube in a bath at 37.5°. At the end of a second 5-minute interval remove the tube from the bath and read at 650 mμ against a reagent blank. Continue to make a series of readings at 5-minute intervals until a maximal density value is reached.

Cholesterol and 7-dehydrocholesterol. The sample is a residue on evaporation. Dry for 2 hours *in vacuo.* Dissolve in 2 ml. of glacial acetic acid and cool. Add 4 ml. of cooled fresh 5 per cent sulfuric acid in acetic anhydride. Store at 25° and read at 620 mμ periodically until a maximum is passed. The usual times for reaching maxima are pure cholesterol 30-35 minutes, 7-dehydrocholesterol or ergosterol 1.5 minutes, α-7-hydroxycholesterol or β-7-hydroxycholesterol somewhat more slowly.

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[53] Albert Zlatkis, Bennie Zak, and Albert J. Boyle, *J. Lab. Clin. Med.*, **41**, 486-92 (1953).

For mixtures of cholesterol and 7-dehydrocholesterol calculate from the readings at 1.5 and 33 minutes.

R_1 = reading in L units at 1.5 minutes
R_2 = reading in L units at 33 minutes
X = L value for 1 mg. of 7-dehydrocholesterol in 6 ml. of reacting solution at 1.5 minutes
y = L value for 1 mg. of cholesterol in 6 ml. of reacting solution at 33 minutes.
a = L value for 1 mg. of cholesterol in 1.5 minutes
b = L value for 1 mg. of 7-dehydrocholesterol in 33 minutes
C = mg. of cholesterol in 6 ml. reacting
D = mg. of 7-dehydrocholesterol in 6 ml. reacting

$$R_1 = Ca + DX$$
$$R_2 = Cy + Db$$
$$D = (R_1y - R_2a)/(xy - ab)$$
$$C = (R_2x - R_1b)/(xy - ab)$$

Usually

$$C = 0.755R_2 - 0.388R_1$$
$$D = 0.207R_1 - 0.002R_2$$

As pyridyl cholesteryl sulfonate. To 0.5 ml. of carbon tetrachloride solution of sample add 0.1 ml. of redistilled pyridine and then 0.25 ml. of a saturated solution of chlorosulfonic acid in carbon tetrachloride. Mix and shake occasionally for 10 minutes. Add 5 ml. of hexane, shake, and centrifuge. Discard the liquid layer and wash the precipitate with 4 ml. of hexane. Dry the deliquescent precipitate in nitrogen and dissolve in 1 ml. of acetic acid at not over 60°. Add 3 ml. of fresh 1:25 concentrated sulfuric acid-acetic anhydride. After 34 ± 5 minutes at 25° read at 625 mμ against a reagent blank.

By zinc chloride in glacial acetic acid.[54] Prepare a reagent by dissolving 80 grams of zinc chloride in 320 grams of glacial acetic acid by shaking. It must not precipitate with chloroform, nor give a precipitate with the sample in chloroform. To 5 ml. of chloroform solution of the sample add 1 ml. of the reagent and 1 ml. of acetyl chloride. Warm at 70° for 10 minutes, cool, dilute to a known volume with chloroform, and read.

By bromine and Fast Green FCF. To a sample containing 0.1-0.3 mg. of cholesterol in water, carbon tetrachloride, or acetic acid add 1 ml. of

[54] Svend Görtz, *Biochem. Z.* **289**, 313-19 (1937).

94.5 per cent acetic acid. Add 0.125 ml., accurately measured, of per cent solution of bromine in carbon tetrachloride. Mix and afte minutes add 0.15 ml., also accurately measured, of 0.1 per cent solu of Fast Green FCF in 1:17 sulfuric acid. Mix and let stand for a 2 minutes. Mix 150 ml. of 1:2.6 sulfuric acid and 245 ml. of 40 per sodium hydroxide solution and dilute to 1 liter. For use add 1 ml this and 0.15 ml. of 1:17 sulfuric acid to water and dilute to 10 with water. Adjust the pH to 1.7 with a glass electrode. Add 1 ml this to the sample, dilute to 10 ml. and read at above 650 mμ. Estir the excess dye from reading under similar conditions. Each mol cholesterol absorbs 2 atoms of bromine.

Ergosterol

Ergosterol can be read directly at 262, 271, 281, and 293 mμ, but will not differentiate it from vitamin D_3.[55] Numerous other compou show absorption in that range. The antimony trichloride reaction vitamins A (Vol. III, page 89) and D (page 380) has been modi for application to ergosterol, cholesterol, and 7-dehydrocholesterol, the latter case to differentiate vitamins D_2 and D_3. When a chlorofo solution of ergosterol is treated with antimony trichloride and ace chloride in chloroform, the yellow color developed has a maximum 393 mμ reached in less than 2 minutes, which remains substantia constant for at least 7 minutes. If the ergosterol is not pure, a reddi yellow color is obtained. The method is better than the direct ultravio absorption method because it differentiates ergosterol from 7-dehyd cholesterol. It has been applied to determination of ergosterol in yea plant extracts, and sterol mixtures. It can be adapted to the quantitat determination of cholesterol, 7-dehydrocholesterol, and other stero Variation in the range of room temperature does not affect the resu Traces of moisture and polar solvents must be rigorously excluded.

A related method [56] uses antimony chloride and acetic anhydride give a blue color of low extinction at 680 mμ. Methyl dichloroarsine a gives a yellow color with ergosterol in chloroform.[57] The blue with t

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[55] T. R. Hogness, A. E. Sidwell, and F. P. Zscheile, *J. Biol. Chem.* **120**, 239-2 (1937); W. Huber, G. W. Ewing, and J. Kriger, *J. Am. Chem. Soc.* **67**, 609 (1945).

[56] Helene Goldhammer and Franz M. Kuen, *Biochem. Z.* **267**, 406-16 (1933).

[57] P. M. Baranger and J. M. Mercier, *Biochem. J.* **36**, 703-5 (1942).

chloroacetic acid[58] does not conform to Beer's law, gives only approximate results, and increases with time.[59]

Ergosterol in carbon tetrachloride gives a stable orange color with green fluorescence on treatment with sulfuric acid.[60] A constant color is reached in 50 minutes, suitable for reading at 550 mμ.

Procedure—*By antimony trichloride.* To prepare the reagent[61] dissolve 18 grams of anhydrous antimony trichloride in 100 ml. of highly purified chloroform (Vol. III, page 80) at 35-45°. Filter the solution and add 3 ml. of acetyl chloride. If stored in a brown bottle, this is stable for at least 1-2 weeks.

The sample solution should contain 0.05-0.5 mg. of ergosterol per ml. in chloroform. For development of color,[62] mix 1 ml. of sample solution with 10 ml. of the reagent. Read at 393 mμ with a slit width of 0.068-0.072 mm. exactly five minutes after the reagent is added. The $E^{1\%}_{1\ cm.}$ is 250.

By sulfuric acid. To a sample containing around 1 mg. of ergosterol per ml. in carbon tetrachloride, add 10 ml. of concentrated sulfuric acid. Mix by inverting several times. Color will form in the lower layer. Read this layer at 550 mμ.

7-Dehydrocholesterol

The digitonide of 7-dehydrocholesterol is quantitatively precipitated if as much as 49 per cent of water is present.[63] When split the sterol can be read at 281.5 mμ.[64] A 25 per cent solution of mercuric acetate in concentrated nitric acid causes absorption at 370 mμ and is specific for this sterol and similar homoannular polyenes.

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[58] Otto Rosenheim, *Ibid.* 23, 47-53 (1929); Irvine H. Page, *Biochem. Z.* **220**, 420-31 (1930); F. Bilger, W. Halden and M. K. Zacherl, *Mikrochemie* **15**, 119-40 (1934).

[59] V. V. Oppel and E. A. Markaryan, *Proc. Sci. Inst. Vitamin Research* USSR, I, 173-87 (1937).

[60] R. B. Petersen and E. H. Harvey, *Ind. Eng. Chem., Anal. Ed.* **16**, 495-6 (1944).

[61] Cyril H. Nield, Walter C. Russell, and A. Zimmerli, *J. Biol. Chem.* **136**, 73-79 (1940).

[62] Frances W. Lamb, Alexander Mueller, and George W. Beach, *Ind. Eng. Chem., Anal. Ed.* **18**, 187-90 (1946).

[63] J. W. Winkert, B.S. Thesis, Polytechnic Institute of Brooklyn (1951).

[64] Albert E. Sobel, Morris Goldberg, and Solomon R. Slater, *Anal. Chem.*, **25**, 629-35 (1953).

An alternative is to use the procedure for ergosterol by antimony trichloride except that the solution of sample should contain 0.03-0.3 mg. of 7-dehydrocholesterol per ml. Read at 322 mμ 8 minutes after the addition of reagent. The $E^{1\%}_{1\,\text{cm.}}$ is 320 at 322 mμ.

Sample—*Serum.* Mix 2 ml. of serum with 25 ml. of 2.8 per cent alcoholic potassium hydroxide solution. Incubate at 60° for about 75 minutes. Transfer to a stemless separatory funnel, rinsing the saponified sample in with 15 ml. of water followed by 15 ml. of 30-40° petroleum ether. Shake 2-3 minutes with occasional venting of pressure. Remove the petroleum ether layer and re-extract the aqueous layer with 10 ml. more of petroleum ether. Evaporate the combined solvent extracts in a conical centrifuge tube at 35° under a stream of nitrogen.

Dissolve the unsaponifiable lipoids in 3 ml. of 1:1 acetone-ethanol. Prepare a solution of 1 gram of digitonin in 55 ml. of alcohol and 45 ml. of water at about 60° and let it cool. Add 1.5 ml. of the digitonin reagent and stopper for 3 hours. Prepare a solution of 100 mg. of digitonin in 27 ml. of water at 60° and let cool. Add 2.7 ml. of this solution and after mixing stopper for 15 hours. Centrifuge to settle the digitonides and aspirate the upper layer.

Suspend the impure digitonides in 10 ml. of 1:1 dioxane-ether. Centrifuge and aspirate the wash solution. Repeat once more with dioxane-ether and twice with ether alone. Finally dry the residue in the tube at 45° under nitrogen.

Add 0.5 ml. of pyridine to the dried residue and incubate at 60° for 30 minutes to split the digitonides. Add 10 ml. of 30-40° petroleum ether and swirl until the pyridine is fully dissolved and the digitonin suspended. Centrifuge and recover the petroleum ether layer. Repeat with 5 ml. of petroleum ether. Evaporate the combined extracts under nitrogen at 35°. To remove traces of pyridine, redissolve the sterols in 3 ml. of petroleum ether and re-evaporate.

Procedure—Dissolve the sterol residue in 5 ml. of aldehyde-free ethanol. Read at 281.5 mμ. As reagent for a second method of reading, dissolve 25 grams of mercuric acetate in 100 ml. of concentrated nitric acid and add a few crystals of urea. Stored in the dark this keeps for several months. Mix 4 ml. of sample solution and 1 ml. of this reagent. Incubate in the dark at 25° for 30 minutes and read at 370 mμ against a blank of reagent and ethanol.

7-Hydroxycholesterol

A blue color is given by 7-hydroxycholesterol with glycerol dichlorohydrin.[65] The digitonide of this sterol is quantitatively precipitated in the presence of not less than 59% of water.[66] The determination has been applied in detail to serum.[67] There is no interference by 7-dehydrocholesterol or by bile acids.

Sample—*Serum.* Prepare exactly as described for 7-dehydrocholesterol. This yields mixed sterols from a 2-ml. sample in dry form.

Procedure—As reagent distil glycerol dichlorohydrin over 1 per cent of antimony trichloride at 10-30 mm. pressure. Discard the first fraction and retain as reagent the 93-108° distillate which should be colorless.

Dissolve the sterol residue in 1 ml. of glacial acetic acid and add 4 ml. of the reagent. Incubate in the dark at 25° for 30 minutes and read at 590 mμ against a reagent-acetic acid blank.

Phytosterol

The general reaction of sterols with acetic anhydride and sulfuric acid is applicable in the absence of cholesterol to estimation of phytosterol.[68] The maximum color is developed in 10 minutes and begins to fade about 5 minutes later. The reaction of cholesterol in acetic acid solution with acetyl chloride and anhydrous zinc chloride is a general reaction which is also applicable to solutions of phytosterol in glacial acetic acid.[69]

Sample—*Meal.* Dry the sample in the form of small pellets or strips. Extract a suitable weight of sample with ether for 6 hours. Evaporate the ether and dry the residue at 100°. Dissolve the residue in acetone and ethyl acetate, either separately or together. Filter if necessary and dilute to a known volume.

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65 Albert E. Sobel, P. S. Owades and J. L. Owades, *J. Am. Chem. Soc.*, **71**, 1487-9 (1949).

66 J. W. Winkert, B. S. Thesis, Polytechnic Institute of Brooklyn (1951).

67 Albert E. Sobel, Morris Goldberg, and Solomon R. Slater, *Anal. Chem.*, **25**, 629-35 (1953).

68 H. Riffart and H. Keller, *Z. Untersuch. Lebensm.* **68**, 113-38 (1934).

69 August L. Bernoulli, *Helv. Chim. Acta* **15**, 274-86 (1932).

Procedure—*By acetic anhydride and sulfuric acid.* Mix a suitable aliquot of the sample with ethyl acetate to give a volume of about 10 ml. Filter if necessary and dilute to 20 ml. with the same solvent. Mix 9 ml. of ethyl acetate and 4 ml. of acetic anhydride. Add 0.8 ml. of concentrated sulfuric acid and shake for 10 minutes. Cool to 20°. Add 1 ml. of the sample, mix, and shake. Let stand for 10 minutes at 20° and read.

By acetyl chloride and anhydrous zinc chloride. Follow the procedure for cholesterol (page 373).

Coprosterol

Coprosterol, which is present in feces, is estimated in substantially the same way as cholesterol by acetic anhydride and sulfuric acid.[70]

Sample—*Feces.* Add 1 gram of calcium hydroxide to 2-3 grams of well-mixed moist feces and mix. Add 10 ml. of 20 per cent sodium hydroxide solution. Mix and heat on a water bath for about 2 hours with stirring. When nearly dry, remove and add 3-4 grams of finely divided plaster of Paris. Mix and dry for 2 hours at 95°. Extract the residue with chloroform and dilute the extract to 25 ml.

Procedure—Follow that for cholesterol (page 371) and report in terms of a cholesterol standard. The amount of color given by coprosterol is not the same as that from cholesterol so that this gives only a relative comparison between different extracts.

Sitosterols

The method of estimation of sitosterols in leaf meal also determines *spinasterol*.[71] The reaction is the general one with acetic anhydride and sulfuric acid. The main problem is in preparation of the sample solution. Thus, the preparation consists of extraction, removal of chlorophyll and xanthophylls by sorption, precipitation of carotene by iodine, precipitation of the sterols as the digitonide, and finally development of the dissolved digitonide in chloroform by acetic anhydride and sulfuric acid. Precipitation with digitonin is equally as applicable to plant sterols as to those of animal origin.

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[70] V. C. Myers and E. L. Wardell, *J. Biol. Chem.* 36, 147-56 (1918); J. A. Gardner and M. Williams, *Biochem. J.* 15, 363-75 (1921).

[71] Monroe E. Wall and Edward G. Kelley, *Ind. Eng. Chem., Anal. Ed.* 19, 677-83 (1947).

The colorimetric reaction is given only by unsaturated sterols. The total sterols can be determined gravimetrically and saturated sterols determined by difference. The precision is better than ±5 per cent. While the color develops differently with sterols from different sources, it reaches a constant value at 30 minutes in all cases. The standard must be sterols from the same source as the sample.

Sample—*Leaf meal.* Extract a 25-gram sample of 30-40 mesh material at 4-8 per cent moisture with petroleum ether in an all-glass Soxhlet.

Free sterols. Prepare a 7 × 2 cm.-sorption column of 3 parts of Hyflo Super-Cel and 1 part of activated magnesia.[72] Sorb a 5 or 10-ml. aliquot of the extract and elute the sterols and carotenes with 5 per cent acetone in low-boiling petroleum ether. Xanthophyll and chlorophyll are retained by the column. Evaporate the extract to 5-10 ml. on a water bath.

Precipitate carotenes as the insoluble iodide by adding 5 ml. of a 0.2 per cent solution of iodine in petroleum ether. Filter on a thin layer of Super-Cel in a small sintered-glass filter and wash with precooled petroleum ether. Shake the filtrate with 5 ml. of 10 per cent sodium thiosulfate solution to remove excess iodine and wash the filtrate successively with 10 ml. of water and 10 ml. of 90 per cent methanol. Evaporate the colorless to light-yellow solution to about 10 ml. and dilute to 25 ml. with petroleum ether.

Evaporate a 5-ml. or 10-ml. aliquot in boiling water. Add 4 ml. of a 2 per cent solution of digitonin in ethanol, stir with a rod and evaporate to dryness. Remove the last traces of moisture with air and wash with 7-8 ml. of petroleum ether. Remove the stirring rod with adhering precipitate and save. Centrifuge and decant. Wash the rod and precipitate again and finally dry by heat and suction. Warm and stir to dissolve the residue from the rod and tube in 2 ml. of glacial acetic acid for determination with acetic anhydride and sulfuric acid.

Total sterols. Reflux a 5-ml. or 10-ml. aliquot of the extract for 30 minutes with 5 ml. of 10 per cent potassium hydroxide in ethanol. Transfer to a separatory funnel alternately with small amounts of ethanol and petroleum ether and add sufficient water to cause separation of the layers. Remove the lower layer and extract with 3 successive

[72] Monroe E. Wall and Edward G. Kelley, *Ibid.* **15**, 18-20 (1943); *Ibid.* **18**, 198-201 (1946).

portions of petroleum ether. Extract the combined petroleum ether layer and washings with 5 successive portions of 90 per cent methanol to remove xanthophyll, traces of chlorophyll, and alkali. Evaporate to 5-10 ml. and complete as for free sterols, starting at "Precipitate carotenes as the insoluble iodide . . . ."

Combined sterols. Subtract free sterols from total sterols.

Procedure—Adjust 2 ml. of an acetic-acid solution of sample containing 0.2-0.8 mg. of sterols to 24° and add 4 ml. of acetic anhydride. When well-mixed, similarly mix in 0.2 ml. of concentrated sulfuric acid. After 30 minutes read at 620 mμ against a reagent blank containing 4 ml. of 0.2 per cent digitonin. The standard curve must have been obtained with sterols from the same source as the sample.

Vitamin D

While the term vitamin D is used as a general description, vitamin D_2 is calciferol or irradiated ergosterol and D_3 is irradiated 7-dehydrocholesterol. The difference between the two is a double bond in D_2 not present in D_3. The maximum absorbency is at about 265 mμ. The extinction coefficients, $E^{1\%}_{1\ \text{cm.}}$ are 500 for D_3 and 460 for D_2. While direct reading may be used,[73] the vitamin A band at 328 mμ interferes. Cartinoids also interfere.

The most widely used and official method is by rat assay. Of the colorimetric methods, that of development of color with antimony trichloride is used most. Antimony trichloride and vitamin D_2 or D_3 in anhydrous solution give maximum absorption at 500 mμ. The color values for vitamins D_2 and D_3 are identical. The reagent solution may be antimony trichloride in chloroform,[74] that plus sulfuric acid, the latter plus acetyl chloride,[75] antimony trichloride in acetic anhydride.[76]

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[73] E. Reerink and A. van Wyk, *Chem. Weekblad* 29, 645 (1932); L. Fuchs and Z. Beck, *Pharm. Presse, Wiss prakt. Heft.* 38, 81-7 (1933); H. Töpelmann and W. Schuhknecht, *Z. Vitamin forsch.* 4, 111-20 (1935); Hans Brockmann, *Z. physiol. Chem.* 241, 104-15 (1936); Hans Brockmann and Ameliese Busse, *Ibid.* 256, 252-71 (1938); E. Marcussen, *Dansk. Tids. Farm.* 13, 141-5 (1939); B. Ol Khin, *Proc. Sci. Inst. Vitamin Research USSR* 3, 28-9 (1941); V. Lasareff and E. V. Rouir, *Bull. soc. chim. Belges* 56, 369-77 (1947).

[74] Hans Brockmann and Yun Hwang Chen, *Z. physiol. Chem.* 241, 129-33 (1936).

[75] C. Nield, W. Russell, and A. Zimmerli, *J. Biol. Chem.* 136, 73-9 (1940); A. Zimmerli, C. H. Nield, and W. C. Russell, *Ibid.* 148, 245-6 (1943).

[76] Y. Raoul and P. Mennier, *Compt. rend.* 209, 546-8 (1939).

or the latter plus sulfuric acid. Another form of the reagent [77] is 20 per cent antimony trichloride and 5 per cent guaiacol in chloroform.

With acetyl chloride present in the reagent, accuracy within 3 per cent can be attained,[78] as its presence makes the reaction about 3 times as sensitive. As little as 0.0002 mg. is determined accurately by the difference between absorption at 500 and 550 mμ.

Various methods for removal of interfering materials are applied. Sterols are removed with digitonin. Vitamin A is removed [79] by sorption on aluminum oxide,[80] but the reaction is still not applicable to fish-liver oils or to products of irradiation of the provitamins. A chromatographic process is inapplicable in many cases.[81] Sorption from benzene is not fully satisfactory unless sterols are removed by digitonin.[82] Vitamin A can be removed with maleic anhydride, followed by separation of sterols by freezing.[83]

In general the concentrations of antimony chloride and acetyl chloride are varied over wide ranges without change in sensitivity. Ethylene dichloride increases the sensitivity. With one version of the reagent [84] the relative $E^{1\%}_{1\,\text{cm.}}$ is for vitamins D_2 and D_3 1800 at 500 mμ, for sterols with 1 double bond 2.2 at 500 mμ, for sterols with 2 double bonds 7 at 515 mμ. Using 10 ml. of a chloroform solution of antimony chloride, saturated at 20°, to 1 ml. of sample solution, the color conforms to Beer's law only below 0.005 mg. per ml.; additional increments give less color.[85] At 30° the color reaches a maximum in 4 minutes and fades thereafter. Development in bright light gives results 15 per cent low, in shaded area 10 per cent low. The maximum color development is at 42°. Between 19° and 33° a 40 per-cent difference occurs in the color developed.

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[77] Willibald Diemair and Gisela Manderscheid, *Z. anal. Chem.* **129**, 254-66 (1949); *Deut. Lebensm. Rundschau* **44**, 240 (1948).

[78] Poul Bonnén Nielsen, *Dansk. Tids. Farm.* **23**, 21-54 (1949).

[79] A. Emmerie and M. van Eekelen, *Acta Breira Neerland. Physiol. Pharmacol. Microbiol.* **6**, 133-6 (1936); D. T. Ewing and F. Tompkins, *Michigan State College Ph.D. thesis*, 1942; I. N. Garkina and V. N. Bukin, *Biokhimiya* **16**, 176-85 (1951).

[80] K. Ritsert, *Merck's Jahresber.* **52**, 27-38 (1938).

[81] J. Hage, M.S. thesis, Michigan State College, 1943; R. Young, *Ibid.* 1943; M. J. Powell, *Ibid.* 1946; M. H. Baker, *Ibid.* 1949.

[82] L. K. Wolff, *Z. Vitamin forsch.* **7**, 277-9 (1938).

[83] M. A. Gudlet, *Proc. Sci. Inst. Vitamin Research USSR* **3**, No. 1, 35-42 (1941).

[84] D. T. Ewing, M. J. Powell, R. A. Brown, and A. D. Emmett, *Anal. Chem.* **20**, 317-20 (1948).

[85] Edgar M. Shantz, *Ind. Eng. Chem., Anal. Ed.* **16**, 179-80 (1944).

Glycerol dichlorohydrin or related compounds react with vitamins D_2 and D_3 in the presence of acetyl chloride or other acid halides.[86] The reaction of vitamin D_2, calciferol, is to give an immediate yellow color which turns green in 1 minute and reaches a maximum at 625 mμ in 15 minutes. This color is stable for several hours. Ergosterol gives a faint pink color at once which turns to orange in 15-20 minutes and later is altered to a fluorescent green. With vitamin D_3 or 7-dehydrocholesterol there is no immediate color development, but later a pink appears and intensifies for at least 24 hours. Cholesterol gives no color. By reading vitamins D_2 and D_3 at 625 mμ there is less than 4 per cent of interference by ergosterol and less than 0.3 per cent by 7-dehydrocholesterol.

The color-producing reagent and the colors produced are relatively stable. The form of the absorption curves is distinctive for vitamin D with a maximum between 500 and 525 mμ. These maxima are further apart than with antimony trichloride and as a corollary there is less interference by the provitamins.

The reaction is suitable for application to products of irradiation of ergosterol.[87] Toxisterol and the suprasterols are assumed to be absent. Ergosterol and lumisterol do not interfere significantly. Tachysterol interferes, but treatment with maleic or citraconic anhydride forms a compound with no significant coloration in the range read. Maleic anhydride is inferior as a reagent because it gives a yellow color.

Vitamin D gives a deep green color with aluminum chloride in anhydrous solution.[88] Addition of pyrogallol converts the color to a red-violet. By dissolving in absolute ethanol this can be used for colorimetric estimation.[89] The color is not given by phytosterol, ergosterol, cholestrol, or lumisterol. Vitamin A and carotenoids must be absent. Suprasterol II gives a similar but weaker reaction. The reaction is carried out before addition of ethanol because prolonged heating of pyrogallol and aluminum chloride in absolute ethanol gives a pink color. Unsaponified fat must be absent. Solvents other than petroleum ether, benzene, chloroform, and absolute ethanol must be absent.

Vitamin D in trichloroacetic acid gives a rose-violet when reacted

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[86] Albert E. Sobel, A. Margot Mayer, and Benjamin Kramer, *Ibid.* **17**, 160-5 (1945).

[87] G. Pirlot and E. V. Rouir, *Bull. soc. chim. Belges* **56**, 296-308 (1947).

[88] Hariklia Tzóni, *Biochem. Z.* **287**, 18-22 (1936).

[89] Wilhelm Halden and Hariklia Tzóni, *Nature* **137**, 909 (1936); Wilhelm Halden, *Naturwissenshaften* **24**, 296-7 (1936).

with furfural.[90] The essentials of the reaction are an energetic dehydrating agent and a strong acid.[91] Therefore, it is carried out in ethanol with furfural and sulfuric acid. The reaction will determine calciferol in the presence of vitamin A. At high concentrations the color becomes blue.

Samples—*Oils.*[91] Weigh a sample of 0.5-2 grams of oil or 0.1 gram of concentrate containing 4,000-100,000 units. For samples up to 1 gram add 10 ml. of 2.8 per cent potassium hydroxide in ethanol; for more than 1 gram add 10 ml. per gram. Heat with occasional agitation at 70-75° with a simple condenser, such as a funnel in the mouth of the flask, until saponification is complete, usually about 1 hour. Add water equal to double the volume of alkali solution to the cooled sample and make 4 successive extractions with 25-ml. portions of ether. Gentle shaking will not form emulsions; if they are formed, break with a few drops of ethanol. Wash the combined ether extracts with 3 successive 50-ml. portions of water and discard the washings. If emulsions form in this extraction of soap, break with a couple of ml. of ethanol. Add 25 ml. of water, shake vigorously, add 25 more ml. of water, and let separate. Discard this washing and repeat twice; more if the final layer is not neutral to phenolphthalein.

Filter the washed ether extracts through paper containing anhydrous sodium sulfate. Rinse the separatory funnel and paper with 25 ml. of ether to remove the last vitamin D. This ether solution can be transformed into a solution suitable for chromatographing in some technics, or evaporated and taken up in a solvent for direct color development by others.

Cromatographic separation.[93] Fit a 2 × 10-cm. Allihn sugar tube with a fritted column of medium porosity. Apply suction and pack with a mixture of equal parts of reagent-grade magnesia and diatomaceous earth to a depth of 6 cm. Add 1 cm. of anhydrous sodium sulfate well packed.

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90 Maurice Pesez, *Bull. soc. chim. France* **1949**, 507-8.

91 J. L. Fontán Candela, V. Villar Palasí, and A. Santos Ruiz, *Anales real. soc. españ. fis. y quim.* **46B**, 509-16 (1950).

92 D. T. Ewing, G. V. Kingsley, R. A. Brown, and A. D. Emmett, *Ind. Eng. Chem., Anal. Ed.* **15**, 301-5 (1943); Cf. Akiji Fujita and Masataro Aoyama, *J. Biochem.* (Japan) **37**, 113-27 (1950).

93 James B. DeWitt and M. X. Sullivan, *Ind. Eng. Chem., Anal. Ed.* **18**, 117-19 (1946).

Evaporate the ether from the unsaponifiable portion of the sample and take it up in a minimum volume of petroleum ether. Wet the column with 50 ml. of petroleum ether, add the sample, and develop the chromatogram with successive 5-ml. portions of petroleum ether. Observation in low-intensity ultraviolet light [94] shows five bands: (1) a narrow intensely pale-blue band, (2) a broad greenish-yellow band containing vitamin A, (3) a narrow light-gray band, (4) two narrow bluish bands about 2 mm. apart, and (5) a narrow gray-blue band of the sterol fraction. Collect the eluate from zones (3), (4), and (5) separately and evaporate the solvent. Take up each in petroleum ether and dilute each to the same volume with petroleum ether, a volume at which zone 4 should contain approximately 1,000 units of vitamin D per ml. Develop the color by the procedure for solutions purified by chromatography.

Capsules. Remove the contents of not less than 5 capsules quantitatively and proceed as described for oils.

Tablets. Pulverize in a mortar and boil a portion containing 4,000-100,000 units with 10 ml. or more of 2.8 per cent ethanolic potassium hydroxide for 30 minutes. Centrifuge out the solids and reheat them with a fresh portion of the ethanolic potassium hydroxide. Again separate. Treat the ethanolic potassium hydroxide solution as described for oils, starting at "Add water equal to double the volume . . .". Wash the solid residue with ether and add it to the ether extract from the solution.

In volatile solvents. Evaporate a solution, such as that of irradiated ergosterol, to dryness under gentle suction at about 50°. Take up in a solvent selected according to the form of the reagent to be used.

Removal of vitamin A and carotenoids with maleic anhydride.[95] Remove the last traces of water from the unsaponifiable extract by distillation of a mixture of benzene and absolute ethanol from it. Dissolve the residue in absolute methanol, cool to −10 to −15°, and filter at that temperature to remove the greater part of the sterols. Evaporate the filtrate and take up in 5 ml. of peroxide-free 1,4-dioxane. Add about 2 ml. of a 10 per cent solution of maleic anhydride in dioxane. It is preferable that the reagent be prepared from fresh maleic anhydride

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[94] J. B. Wilkie and J. B. DeWitt, *J. Assoc. Official Agr. Chem.* **28**, 174-86 (1945).

[95] Nicholas A. Milas, Robert Heggie, and J. Albert Raynolds, *Ind. Eng. Chem., Anal. Ed.* **13**, 227-31 (1941).

made by distilling a 1:1.5 mixture of maleic acid and phosphorus pentoxide.

Heat at 100° for 1 hour after adding the maleic anhydride reagent and cool. Add 10 ml. of 2.8 per cent potassium hydroxide in ethanol and let stand for 5-10 minutes. Add 15 ml. of water and extract with several portions of ether. Wash the ether extracts twice with water and dry with anhydrous sodium sulfate. This ether solution is then transformed into a solution in a solvent appropriate to the technic to be applied.

Procedure—*By antimony trichloride. Solution purified by chromatography.* As reagent mix 20 grams of antimony trichloride and 10 grams of anhydrous calcium chloride. Pulverize in a mortar and shake with 100 ml. of ethylene dichloride boiling at 83-4°. Filter through anhydrous sodium sulfate and add 2 ml. of acetyl chloride per 100 ml. The reagent may be used after 30 minutes and is stable for 10 weeks.

Mix 0.5 ml. of sample solution containing vitamin D in petroleum ether with 9.5 ml. of the reagent and shake vigorously. Read the $E^{1\%}_{1\,cm.}$ at 500 mμ at 30 seconds after adding the reagent and every minute for 5 minutes. Vitamin D gives the maximum color within a minute and remains constant for 5-10 minutes. Sterols react slowly at first but reach their maximum within 5 minutes. The potency is the $E^{1\%}_{1\,cm.}$ due to vitamin D multiplied by 18,200 as a factor.

Alternative. As sample take 1 ml. of solution in chloroform containing 0.07-0.25 mg. of vitamin D_2. Adjust to 30° and add 10 ml. of chloroform saturated with antimony trichloride at 20°, then warmed to 30°. After 4 minutes at 30° in the dark, read at 500 mμ.

By glycerol dichlorohydrin. To 0.02 gram of the product of irradiation of ergosterol add, in an atmosphere of carbon dioxide, 2 ml. of a 0.7 per cent solution of citraconic anhydride in benzene. After 30 minutes, evaporate the benzene and take up in 50 ml. of chloroform. Mix a 3-ml. portion of this with 2 ml. of glycerol-1,3-dichlorohydrin (Vol. III, page 90), shake, and let stand in the dark for 26 minutes. Read the color for vitamin D at 625 mμ. It is stable for at least 2 hours.

By pyrogallol and aluminum chloride. Select a volume of sample in benzene, petroleum ether, or chloroform which will contain 0.01-0.1 mg. Add 0.5 ml. of a 0.1 per cent solution of pyrogallol in absolute ethanol. Evaporate in boiling water to a volume of approximately 0.5 ml. Add 2-4 drops of a fresh 10 per cent solution of anhydrous aluminum

chloride in absolute ethanol. Heat for a total of 4 minutes to develop the maximum color. Dilute with absolute ethanol to a suitable volume and read.

By furfural. To a sample in ethanol add 0.5 ml. of 1 per cent furfural in ethanol and dilute to 2.5 ml. with ethanol. Add 1 ml. of concentrated sulfuric acid dropwise over a period of 1 minute. After 2 minutes add 3.5 ml. of 0.4 per cent sulfuric acid in ethanol over a period of 1 minute. Mix and, after exactly 2 minutes, read photometrically.

CHAPTER 10

HORMONES [1]

In the organization of slightly over 20 hormones, members of at least four classes, and with well over 100 names, there are problems. Therefore, they are presented according to a common, if not the most commonly used, name with numerous equivalents following in many cases.

The type reaction of the 17-ketosteroids or androgens is a pink color with *m*-dinitrobenzene. 2,4-Dinitrophenylhydrazine, 3,5-dinitrobenzoic acid, and *m*-dinitrophenol also react. Many have other reactions depending on characteristic configurations. Similarly a type reaction of 3-ketosteroids or estogens is that with phenol—or β-naphthol sulfonic acid. Many hormones react with *p*-dimethylaminobenzaldehyde, *m*-nitrobenzaldehyde, salicylic aldehyde, and antimony trichloride.

Androsterone, Androstan-3-α-ol-17-one, 3-Hydroxy-androstan-17-one

Androsterone, as a natural male hormone which can also be synthesized, is the structural parent of a group of hormones. It is representative of a class of nonphenolic ketosteroids derived from androstane, called androgens as a class name. The common reaction for its estimation is with *m*-dinitrobenzene in alcoholic alkali to give a pink color.[2] The maximum color occurs with a carbonyl group in position 17 and a

[1] See Chapter 1, Volume III, for details of organization, condensation, etc.

[2] W. Zimmermann, *Z. physiol. Chem.* **233**, 257-64 (1935); *Ibid.* **245**, 47-57 (1936); H. Wu and C. Y. Chou, *Chinese J. Physiol.* **11**, 413-28 (1937); Rudolph Neustedt, *Endocrinology* **23**, 711-17 (1938); Harry B. Friedgood and Helen L. Whidden, *Ibid.* **25**, 919-24 (1939); *Ibid.* **27**, 242-81 (1940); *Ibid.* **28**, 237-47 (1941); *New Engl. J. Med.* **220**, 736-41 (1939); Nathan B. Talbot and George O. Langstroth, *Endocrinology* **25**, 729-36 (1939); Nathan B. Talbot, Allen M. Butler, and E. Machlachlan, *J. Biol. Chem.* **132**, 595-603 (1940); Nathan B. Talbot, Allen M. Butler, E. A. Machlachlan and R. N. Jones, *Ibid.* **136**, 365-77 (1940); N. B. Talbot, John K. Wolfe, E. A. Machlachlan, and R. A. Berman, *Ibid.* **139**, 521-34 (1941); N. B. Talbot, R. A. Berman, E. A. Machlachlan and J. K. Wolfe, *J. Clin. Endocrinol.* **1**, 668-73 (1941); N. B. Talbot, R. A. Berman, and E. A. Machlachlan, *J. Biol. Chem.* **143**, 211-18 (1942).

methylene carbon at position 6.[3] It follows that ketosteroids other than androsterone give a similar reaction. The sensitivity permits detection of 0.2 mg. of androsterone, provided the interfering hormones and acetone are absent. The rapidity of development and absorption spectrum varies with variation in the structure[4] of interfering ketosteroids and is suitable for photometric reading.[5] Variations in concentrations of alcohol, *m*-dinitrobenzene, and potassium hydroxide as well as time of color development modify the result.[6] Spectrophotometric separation of the color of ketonic and nonketonic bodies in this reaction can be applied.[7] Alcoholic potassium hydroxide accentuates the difference in color between the two fractions. Also it makes the color from the nonketonic fraction more linear.[8] The color from aqueous alkali is more stable. The reaction is satisfactory in 4:1 benzene-alcohol.[9] Extraction of the color from an aqueous solution into ether is applicable to avoid interference by pigments.[10] Some deviations from Beer's law occur.[11] The color substances can be extracted into absolute ether.[12] Cloudiness from formation of carbonate in strong alcoholic solutions is thus avoided.

The 17-ketosteroids fraction checks the ketone fraction of extracts[13] as determined by *m*-dinitrobenzene. When chromatographed on alumina and eluted with benzene, dehydroandrosterone is separated and eluted first.[14] Both give the same color with *m*-dinitrobenzene. Accuracy to ± 2 per cent is to be expected.[15] There is negligible interference by stilbestrol, pregnandiol, benzoic acid, Δ^5-cholestene-3,4-dione, α-chol-

[3] H. W. Marlow, *Ibid.* **183**, 167-71 (1950).

[4] Nancy H. Callow, Robert K. Callow, and Clifford W. Emmens, *Biochem. J.* **32**, 1312-31 (1938); Eleanor Saier, Mary Warga, and Robert C. Grauer, *J. Biol. Chem.* **137**, 317-23 (1941).

[5] Henrique Tastaldi, Octávio Lemmi, and Joaquim Lacoz de Moraes, *Anais faculdade med. univ. S. Paulo* **16**, 381-97 (1940).

[6] Ira T. Nathanson and Hildegard Wilson, *Endocrinology* **33**, 189-203 (1943).

[7] William W. Engstrom and Harold L. Mason, *Ibid.* **33**, 229-36 (1943).

[8] M. G. Mellon, *Anal. Chem.* **21**, 3-7 (1949).

[9] Klaus Walter, *Klin. Wochschr.* **30**, 474-5 (1952).

[10] A. Ruppert, *Z. ges. exptl. Med.* **119**, 229-36 (1952).

[11] H. S. Strickler, M. Evelyn Walton, D. A. Wilson, and Margaret Dienes, *Endocrinology* **29**, 545-57 (1941).

[12] Karl Wulfurt, *Tids. Kjemi, Bergvesen Met.* **7**, 127-30 (1947).

[13] William W. Engstrom and Harold L. Mason, *Endocrinology* **33**, 229-36 (1943).

[14] B. M. de Laat, *Acta Brevia Neerland. Physiol., Pharmacol., Microbiol.* **11**, 51-7 (1941).

[15] A. F. Holtorff and F. C. Koch, *J. Biol. Chem.* **135**, 377-92 (1940).

esterol oxide, Δ^5-7-keto-3-hydroxycholenic ethyl ester, Δ^5-3,4-cholestenedione-4-enol acetate, Δ^5-3-acetoxy-7-ketocholenic ethyl ester, Δ^5-3-acetoxy-5-hydroxy-6-ketochelenic ethyl ester, cholesterol, digitonin, β-hydroxybutyric acid, ergosterol, theelol, and 7-oxocholesterol. The order of magnitude of color developed by androsterone, dehydroandrosterone, and theelin is similar. Bile salts do not interfere with determination of free ketosteroid, but they do after the acid hydrolysis necessary for total ketosteroids. Extra extractions decrease the error so introduced. Glucose and urea do not interfere.

Significant amounts of color are furnished by androstan-17-one, $\Delta^{3,5}$ androstadien-17-one, estrone, androstan-17-ol-3-one, cholestanone, testosterone, cholestenone, and its acetate androstane-3,17-dione, Δ^4-androstene-3,17-dione, *epiallo*-pregnan-3-ol-20-one, progesterone, cholestan-3-one, cholestane-3,6-dione, cyclopentanone, androstenetrione, 6- oxocholesterol, Δ^4-3,6-cholestenedione-6-enol ethyl ether, 3-keto-12-hydroxycholanic acid and its ethyl ester, acetoacetic ester, 6-oxo-17-acetotestosterone, dehydrocholic acid, dehydrodesoxycholic acid, desoxycorticosterone, progesterone, cyclohexanone, phenyl methyl ketone, and acetone. The color produces by 3 (α)-hydroxyetio cholan-17-one or by dehydroisoandrosterone is identical.[16]

The 2,4-dinitrophenylhydrazones of various androgens and estrogens are colored and, properly separated, furnish quantitative estimations of androsterone and testerone.[17] Androsterone also reacts with 3,5-dinitrobenzoic acid in the presence of benzyltrimethylammonium hydroxide to give a purple color.[18] The reaction is also given by estrone, progesterone, testosterone, dehydrocholic acid, etc., but not by such nonketones as estradiol. The color conforms to Beer's law.

When heated with a specific concentration of antimony trichloride in nitrobenzene at 100° and diluted with benzene containing traces of acetic anhydride and acetyl chloride, androsterone gives an intense blue color while dehydroisoandrosterone gives a light green.[19] At another concentration of reagent they give reddish-blue of approximately equal intensity.

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[16] Emil J. Bauman, Nannette Metzger, and David B. Sprinson, *Endocrinology* **30**, 518-19 (1942).

[17] F. P. Veitch, Jr., and H. S. Milone, *Ibid.* **158**, 61-5 (1945); P. E. Hilmer and W. C. Hess, *Anal. Chem.* **21**, 822-3 (1949).

[18] Robert P. Tasney and John M. Cross, *J. Am. Pharm. Assoc.* **39**, 660-3 (1950).

[19] G. Pincus, *Endocrinology* **32**, 176-84 (1943); Leland C. Clark, Jr., and Haskell Thompson, *Science* **107**, 429-31 (1948).

When treated in an alkaline medium with *m*-dinitrophenol, the 17-ketosteroids including androsterone and dehydroandrosterone give a red color. When the steroids are chromatographed on alumina and eluted for separation, there are 7 peaks of which 2 are these androgens.[20] Thus the method shows a class rather than the individual members. In the absence of estrone, estradiol, equelin, testosterone, corticosterone, and cholesterol, the color of 0.005-0.25 mg. can be read.[21] The reaction with *p*-dimethylaminobenzaldehyde, *m*-nitrobenzaldehyde, and salicylaldehyde is also given by cholesterol, estrone, estradiol, equelin, testosterone, and corticosterone.

Samples—*Separation of androgens from estrogens.*[22] The general scheme for separation is shown in Figure 15. The sample should approximate 5 mg. of hormones. Transfer the material at step 2 to a 500-ml. separatory funnel and thereafter evaporate from that at step 6 by heating the funnel on a water bath. This minimizes transfers. The end products are a neutral androgenic steroid fraction in ethanol and the estrogenic phenols in aqueous potassium hydroxide solution for separation shown on page 403.

Urine. Variability of results if urine is used directly appears to be due to unidentified interfering nonketonic substances. Therefore, the hormones are extracted.[23] Glucuronides and sulfates must be hydrolyzed before the steroid can be extracted and fractionated.[24] If the urine contains protein it must be removed if total steroids are to be obtained. Coagulation with metaphosphoric acid or ultrafiltration is unsatisfactory.

Adjust 150 ml. of urine to pH 4.5 with acetic acid and dilute to 200 ml. Add 10 ml. of a suspension of kaolin, previously activated by washing with 1:10 hydrochloric acid and then washed acid free. Shake for 5 minutes, store at 5°, and centrifuge. Decant and wash the residue twice. Dilute to 250 ml. and use an aliquot for hydrolysis.

Heat 150 ml., deproteinized if necessary, to boiling under a reflux and then add 22.5 ml. of concentrated hydrochloric acid as rapidly as pos-

[20] L. G. Huis in 't Veld, *Nederland Tijdschr. Geneeskunde* **90**, **II**, 666-71 (1946).

[21] Karl Wulfert, *Acta Chem. Scand.* **1**, 818-32 (1947).

[22] T. F. Gallagher, D. H. Peterson, R. I. Dorfman, A. T. Kenyon, and F. C. Koch, *J. Clin. Invest.* **16**, 695-703 (1937); G. Pincus, *J. Clin. Endocrinol.* **5**, 291-300 (1945); Harry B. Friedgood, Josephine B. Garst, and A. J. Haagen-Smit, *J. Biol. Chem.* **174**, 523-54 (1948).

[23] Wilbur A. Robbie and Robert B. Gibson, *J. Clin. Endocrinol.* **3**, 200-5 (1943).

[24] William T. Beher and Oliver H. Gaebler, *Anal. Chem.* **23**, 118-23 (1951).

SAMPLE	CONTROL
(1) 15 ml. 95 per cent ethanol solution of estrogens + 250 ml. of water + 80 ml. of 1:5 sulfuric acid; hydrolyze 10 minutes at 100°; cool rapidly	15 ml. 95 per cent ethanol + 250 ml. of water + 80 ml. of 1:5 sulfuric acid; hydrolyze 10 minutes at 100°; cool rapidly

(2) Extract with four 75-ml. portions of ether

Aqueous phase discarded

(3) Ether extract of hydrolyzate

(4) Wash with three 10-ml. portions of 9 per cent sodium bicarbonate

Discard washings

(5) Ether extract

(6) Reduce to 3 ml. by steam jacket; add 54 ml. of carbon tetrachloride

(7) Ether-carbon tetrachloride 1:18

(8) Extract with four 25-ml. portions of 5.6 per cent potassium hydroxide

(e)	(d)	(c)	(b)	(a)	(9)
So-called neutral steroid fraction	← Evaporate to dryness on warm water bath with suction; take up residue in 5 ml. of 95 per cent ethanol	Ether-carbon tetrachloride extract (1:18)	← Wash twice with 4-ml. portions of water; add wash to aqueous potassium hydroxide phase	Organic phase (ether-carbon tetrachloride)	Aqueous potassium hydroxide phase of estrogenic phenols

FIG. 15. Extraction and separation of estrogens from androgens

sible. Reflux exactly 7 minutes and chill in ice. Storage in a refrigerator up to 3 days at this point will not alter results. Extract with ether in a continuous extractor, having a capacity of 225 ml. below the return tube. Passage of 14 liters of ether per hour for 2 hours is adequate. Extract the reddish ether extract twice with 15 ml. of 5 per cent sodium bicarbonate solution and discard the washings. This gets rid of acidic impurities.

Extract the ether solution twice with 15-ml. portions of 10 per cent sodium hydroxide solution and set these aside for later acidification and ether extraction as the estrogenic fraction. Wash the ether extract of androgens twice with 25-ml. portions of water and evaporate to dryness. Remove the last traces of water with a stream of nitrogen and avoid overheating. This residue is the crude neutral steroid fraction.

Dissolve the fraction in ether and take an aliquot containing 0.04-0.08 mg. of ketosteroids for development with *m*-dinitrobenzene by the procedure for "Crude neutral steroids."

Alternatively, reflux a 250-ml. sample for 10 minutes with 25 ml. of concentrated sulfuric acid. Add 75 ml. of carbon tetrachloride and reflux for 10 minutes longer. Cool and separate the extract. Wash it successively with 20 ml. of water, 20 ml. of 10 per cent aqueous sodium hydroxide, 20 ml. of water, and 20 ml. of water. After centrifuging to separate the last portion of water, evaporate the extract on a water bath. Warm the residue slightly with 10 ml. of ethanol to dissolve. This solution contains all the neutral 17-ketonic steroids, but estrogens have been eliminated. It is suitable for development with *m*-dinitrobenzene.

Hydrolysis of the urine with hydrochloric acid may affect the 3-β-hydroxyl group of β-ketosteroids so that they are no longer separable from α-ketosteroids by precipitation with digitonin. This may also effect the carbonyl group at C_{17} of Δ^5 unsaturated steroids. This is eliminated by simultaneous heating for hydrolysis and extraction with carbon tetrachloride.

As another technic, hydrolysis by autoclaving for 15 minutes at 15 pounds with sulfuric acid present will give extraction by dibutyl ether from 3-minute shaking at 180 shakes per minute.[25]

Blood. Shake 5 ml. of blood serum with 5 ml. of 10 per cent zinc sulfate solution for 4-5 minutes and add, dropwise, 5 ml. of 2 per cent sodium hydroxide solution. Mix well and centrifuge after 10 minutes.

[25] W. Kenneth Cuyler and Margaret Baptist, *J. Lab. Clin. Med.* 26, 881-4 (1941).

Extract 10 ml. of the clear solution with carbon tetrachloride continuously for 2 hours. Distil the solvent and take up the residue in 2 ml. of ethanol for development as the 2,4-dinitrophenylhydrazone.

Procedure—*By m-dinitrobenzene and alkali. Fractionated samples.* To a 0.2-ml. sample containing 0.02-0.08 mg. of androsterone add 0.2 ml. of 2 per cent solution of *m*-dinitrobenzene in redistilled ethanol. This reagent must be no more than 10 days old. Add 0.2 ml. of 5.0 ± 0.02 *N* aqueous potassium hydroxide solution. Stopper, mix, and store at 25° ± 0.2° for 45 minutes. Add 10 ml. of redistilled ethanol and read at 520 mμ after 3 minutes against a reagent blank in which 0.2 ml. of ethanol replaces the sample. If the sample is colored, correct for a blank in which ethanol replaces the *m*-dinitrobenzene reagent. The color on dilution increases for 2 minutes after dilution and begins to fade after 20 minutes. Use a calibration curve prepared for the specific lot of reagents.

Crude neutral steroids. Evaporate the ethereal aliquot in the cuvet. Cool and add 2 ml. of ethanol, 0.2 ml. of 2 per cent *m*-dinitrobenzene in ethanol, and 0.2 ml. of 28 per cent potassium hydroxide solution. After 45 minutes at 25 ± 0.1° add 10 ml. of ethanol and mix. Read at 440 mμ and 515 mμ. Then for the Coleman Junior spectrophotometer the reading is: micrograms in sample = $(A_{515} - 0.77A_{440})/0.00397$.

As the 2,4-dinitrophenylhydrazone. Reflux 2 ml. of ethanol containing 0.05-0.2 mg. of androsterone with 0.5 ml. of saturated alcoholic 2,4-dinitrophenylhydrazine for 2 hours. Add a drop of concentrated hydrochloric acid and reflux for 2 minutes. Add water dropwise to cloudiness and crystallize at 0°. Centrifuge and wash the precipitate with ethanol and with water. Dissolve in 12 per cent chloroform in benzene and sorb on an alumina column. Wash with the solvent to form two bands. Elute that of unreacted hydrazine with the solvent. Elute the other with chloroform. If other hormones are not present, dilute to 25 ml. with chloroform for development of color in a 2-ml. aliquot.

For separation of other hormones take up the solid in benzene and sorb on an alumina column. Elute with 10 ml. of benzene, then with 50 ml. of 1 per cent acetone in petroleum ether, and finally with chloroform until the washings are colorless. Estrone is left in the column.

Evaporate the solvents from the eluate and take up the residue in benzene. Sorb on a column of florisil. Wash with 5 ml. of benzene and elute with 150 ml. of 20 per cent acetone in petroleum ether. Two bands

separate and andresterone and other androgens are eluted in the first washings. Evaporate the solvents from this and take up in 1.25 ml. of chloroform. Dilute to 25 ml. with ethanol for the use of a 2-ml. aliquot.

Combine the 2-ml. aliquot with 8 ml. of 0.56 per cent alcoholic potassium hydroxide solution. Read at 430 mμ and 460 mμ and calculate the results by the following formula.[26]

C_1 = concentration of androsterone
C_2 = concentration of testosterone
K_1^A = slope of androsterone hydrazone at 430 mμ
K_2^A = slope of testosterone hydrazone at 430 mμ
K_1^B = slope of androsterone hydrazone at 460 mμ
K_2^B = slope of testosterone hydrazone at 460 mμ
D^A = reading of density of mixture at 430 mμ
D^B = reading of density of mixture at 460 mμ

The slopes are obtained by dividing the optical densities of standards at the two wave lengths by the number of micrograms of hydrazone in the volume of standard tested.

$$C_1 = (K_2^A D^B - K_2^B D^A)/(K_2^A K_1^B - K_2^B K_1^A)$$
$$C_2 = (D^A - K_1^A C_1)/K_2^A$$

By 3,5-dinitrobenzoic acid. Dry the sample containing 0.002-0.02 mg. of the hormone in a tube in a nitrogen atmosphere. Add 0.5 ml. of a fresh 0.1 per cent solution of 3,5-dinitrobenzoic acid in 40 per cent aqueous benzyltrimethylammonium hydroxide, Triton *B* for example. Mix and let stand for 10 minutes. Add 10 ml. of water, mix, and read at 530 mμ against a reagent blank.

By antimony trichloride. To the anhydrous steroid sample amounting to 0.05-0.25 mg. add 5 ml. of a reagent containing 26 grams of anhydrous antimony trichloride in 5 ml. of nitrobenzene. Stopper and place in boiling water for 40 minutes. Cool and dilute to 25 ml. with benzene containing 0.025 per cent each of acetic anhydride and acetyl chloride. Read at 670 mμ against a reagent blank. At this wave length dehydroandrosterone gives approximately one-third the intensity of androsterone. A correction may be applied by determining the dehydroandrosterone by antimony chloride in acetic anhydride.

[26] H. W. Knudson, V. W. Meloche, and C. Juday, *Ind. Eng. Chem., Anal. Ed.* **12**, 715-8 (1940).

Dehydroepiandrosterone, Dehydroisoandrosterone, Δ^5-Androsten-3-*trans*-ol-17-one

The stable yellow color developed by heating a dehydroandrosterone solution with sulfuric acid is converted to a stable blue-violet on controlled dilution with water.[27] There is no interference by androsterone, isoandrosterone, and Δ^5-androstene-3(β), 17-diol. A similar color given by Δ^4-androstene-3, 17-dione disappears on a few minute's heating. In the absence of testosterone, desoxycorticosterone, and Δ^4-androstene-3β-17-dione, the color developed with alcoholic sulfuric acid is also read by the procedure for testosterone.

A specific method in the presence of androsterone is by development with antimony trichloride in acetic anhydride.[28]

The reaction for cholic acid in acetic acid with furfural[29] is applicable to estimation of dehydroisoandrosterone.[30] The color does not conform fully to Beer's law, but Δ^5-3(β)-chloroandrosten-17-one, and $\Delta^{3,5}$-androstadien-17-one, which are probably acid hydrolysis products of the test substance, and many other androstene derivatives do. Of these only two are ketonic and probably do not occur in urine. They are Δ^5-3(β)-hydroxy-21-acetoxy-20-ketopregnene and pregnenolone. When nonketonic unsaturated steroids are separated before color development, the result may be read as dehydroisoandrosterone. The blue in strong sulfuric acid in ether is also read at 570 mμ.[31]

Procedure—*By sulfuric acid.* Measure out 0.4 ml. of sample solution containing 0.1-0.3 mg. of test substance. Chill and add 2 ml. of cold concentrated sulfuric acid. Place in boiling water for 90 seconds and then cool in ice water. Dilute to 10 ml. with 1:3 sulfuric acid and read at 600 mμ against a reagent blank.

By antimony trichloride. To 0.05-0.25 of solid anhydrous steroid sample add 5 ml. of a reagent consisting of 38 grams of anhydrous

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27 Wilhelm Dirscherl and Friedrich Zilliken, *Naturwissenshaften*, **31**, 349-50 (1943); *Biochem. Z.* **320**, 57-65 (1949); A. Thiel Nielsen, *Acta Endocrinol.* **1**, 121-32 (1948); Wilhelm Dirscherl and Hans Traut, *Klin. Wochschr.* **30**, 159-65 (1952).

28 Leland C. Clark, Jr., and Haskell Thompson, *Science* **107**, 429-31 (1948).

29 L. H. Schmidt and Hettie B. Hughes, *J. Biol. Chem.* **143**, 771-83 (1942).

30 Paul L. Munson, Mary E. Jones, Philip J. McCall and T. F. Gallagher, *Ibid.* **176**, 73-82 (1948).

31 C. C. Jensen, *Nature* **165**, 321 (1950); Lorenz Hansen, *Endocrinology* **46**, 207-14 (1950).

antimony trichloride in 10 ml. of acetic anhydride. Heat in a boiling water bath for 40 minutes. Cool and dilute to 25 ml. with benzene. Read at 660 mμ against a reagent blank.

By furfural and acetic acid. Evaporate a sample containing 0.01-0.05 mg. of dehydroisoandrosterone to dryness on a water bath with a stream of nitrogen. Dissolve the residue in 0.5 ml. of glacial acetic acid. Add 2 ml. of 0.56 per cent solution of furfural in 1:1 acetic acid. Prepare a sample blank without furfural reagent containing 2 ml. of 1:1 acetic acid and a reagent blank in which the reagent is replaced with 2 ml. of 1:1 acetic acid.

To the successive tubes add 7.5 ml. of 1:1 sulfuric acid at 1-minute intervals. Mix well, place in a water bath at 67° ±0.2° for exactly 12 minutes and transfer to an ice bath for a minute. Read at 660 mμ against the blank. Solutions containing furfural increase in color while standing at room temperature, so that all readings should be made at the same time interval ±10 minutes after removal from the bath. Standards must be run with each series. Subtract the sample extract blank from the value obtained against the reagent blank.

Dihydroandrosterone, 3-*cis*-17-*trans*-Androstandiol

This synthetic hormone is structurally a reduction product of androsterone. Practically it is produced from cholesterol and therefore is not likely to be found as a complex mixture with other hormones. The usual class methods given under androsterone are therefore applicable to its estimation.

Testosterone, Δ^4-Androstene-17-*trans*-ol-3-one, Δ^4-Etiocholen-17-(α)-ol-3-one, 17-Hydroxy-Δ^4-androsten-3-one

Testosterone is structurally closely related to dehydroandrosterone, differing in reversal of the location of the two substituents and different location of the double bond. It is either determined simultaneously with, or in the absence of androsterone as the 2,4-dinitrophenylhydrazone (page 392). The color with 3,5-dinitrobenzoic acid as described for androsterone (page 393) is also applicable. The color of testosterone with or without prior solution in acetic anhydride is read in alcoholic sulfuric acid.[32] The same reaction is given by Δ^4-androstene-3β-17-dione, but this can be removed by prior treatment with nicotinic acid

[32] A. Thiel Nielsen, *Acta Endocrinol.* **1**, 121-32, 362-74 (1948).

hydrazide.[33] A lower intensity of color is given by desoxycorticosterone, and a higher by dehydroandrosterone. Prolonged heating prevents the color from the latter two from interfering.

The color of 0.005-0.25 mg. with *p*-dimethylaminobenzaldehyde, *m*-nitrobenzaldehyde, or salicylic aldehyde is read in the absence of estrone, estradiol, equelin, androsterone, corticosterone, and cholesterol.

Sample—*Oily solutions.*[34] To 15 ml. of heptane add an appropriate sample followed by 10 ml. of 90 per cent ethanol. Shake and transfer the ethanol layer to a second funnel containing 15 ml. of heptane. Shake and similarly transfer the ethanol layer to a third funnel containing 15 ml. of heptane. Separate the ethanol and reserve. Wash each of the three heptane layers sequentially with three 10-ml. portions of 90 per cent ethanol. Combine the ethanolic extracts and discard the heptane layers. Dilute to 100 ml. and evaporate 20 ml. to dryness. Take up in a 2 ml. of 95 per cent ethanol and develop with 2,4-dinitrophenylhydrazine (page 392).

Procedure—*By sulfuric acid.* Treat a chilled sample containing 0.001-0.02 mg. of testosterone with 1 ml. of concentrated sulfuric acid. Place in a boiling water bath for 5 minutes and cool at once in ice water. Add 4 ml. of a mixture of 1 volume of concentrated sulfuric acid and 3 volumes of ethanol. After 0.5 hour at 29° read at 600 mμ against a reagent blank.

17-Methyltestosterone, 17(β)-Methyl-Δ^4-androsten-17(α)-ol-3-one

Methyltestosterone has an α, β unsaturated ketone group at carbons 3 and 4 in ring A and exhibits strong ultraviolet absorption.[35] This is used as one method of analysis. Optical density measurements made at the absorption band due to this configuration are not specific, but are more selective than a method depending on optical rotation.[36] There is a straight-line relationship between concentration and optical density.

Infrared absorption spectrophotometry is a highly specific method because no two compounds have identical infrared absorption spectra.

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33 Leon Valluz and André Petit, *Bull. soc. chim.* **12**, 951-2 (1945).

34 Elna Diding, *Svensk Farm. Tid.* **56**, 3-17 (1952).

35 L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," New York, 3rd Ed. p. 190 (1949).

36 Jonas Carol, *J. Assoc. Official Agr. Chemists* **34**, 572-6 (1951).

Infrared spectra usually have many maxima, but it is possible to make absorption measurements where contaminants have little or no absorption. The difficulty in this method lies in obtaining transparent solvents. Since methyltestosterone is soluble in carbon disulfide, it is used for this determination. An ultraviolet method is sensitive and can be used for very small amounts. Both methods are superior to official USP methods.

Sample—Add a weighed sample of 5-10 mg. of powdered methyltestosterone tablets to 25 ml. of water, followed by 25 ml. of ether and shake carefully for 1 minute. Allow the layers to separate and extract the aqueous phase with 25 ml. and 25 ml. of ether. Discard the aqueous layer. Wash the ether fractions successively with 5 ml. of saturated sodium bicarbonate solution and two 5-ml. portions of water. Discard the aqueous washes, combine the ether extracts, and evaporate to dryness on a steam bath.

Procedure—*Ultraviolet.* Dissolve the residue in ethanol and dilute to 100 ml. with ethanol. Dilute a 10-ml. aliquot to 100 ml. with ethanol and read against ethanol at 241 mμ.

Infrared. Dissolve the methyltestosterone residue in a small amount of chloroform. Evaporate the solvent to dryness on a steam bath, using a current of air. Add 10 ml. of carbon disulfide, stopper immediately, and dissolve by gentle mixing. Read against a blank at 10.7 μ.

Δ^4-Androstenedione-3,17

In the absence of testosterone, desoxycorticosterone, and dehydroandrosterone the reaction with alcoholic sulfuric acid is used. Follow the procedure (page 397) for testosterone. An intense specific red color is produced with this hormone by anhydrous aluminum trichloride and benzoyl chloride in nitrobenzene.[37]

Procedure—Prepare a reagent by heating 10 grams of anhydrous aluminum chloride with 10 grams of benzoyl chloride and taking up with 40 ml. of nitrobenzene. To 0.05-0.25 mg. of solid anhydrous sample add 5 ml. of the reagent and heat in boiling water for 40 minutes. Cool and dilute to 25 ml. with the reagent. Read at 570 mμ against a reagent blank.

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[37] Leland C. Clark, Jr., and Haskell Thompson, *Science* 107, 429-31 (1948).

ESTRONE, THEELIN, FOLLICULIN, $\Delta^{1,3,5}$-ESTRATRIEN-3-OL-17-ONE

Estrone is introduced as the first of the estrogens because of its relation to the androgens by having a keto group, as distinguished from estradiol and estriol to follow.

One basic method of determination is by reaction with phenolsulfonic acid or β-naphthol sulfonic acid.[38] The color developed in the anhydrous solution is yellow to orange with a green fluorescence. When water is added and the mixture reheated, the yellow is converted to pink and the fluorescence reduced. This unstable pink color is masked in urine by a brown which can be corrected only approximately. Omission of the phenolsulfonic acid intensifies the fluorescence.

The same color is given by estriol, estradiol, and androsterone, but not by dehydroandrosterone. It is applied to estrone, estradiol, and estriol by separation of the three.

The characteristic band is at 522 mμ. Brown color from non-specific charring of organic matter from urine will add to the values to make them grossly inaccurate unless read at 420 mμ for correction, a band where the pink from estriol does not occur. The correction is conveniently applied by a nomogram. Also by extraction of the sample some interfering substances are eliminated.

The intensity of the pink color is affected by (1) the composition and amount of reagent used, (2) the time of heating to develop the intermediate yellow color, (3) the amount of water added to the yellow-colored solution, and (4) the time of reheating. The results are accurate to 5 per cent at 5 mg. per liter and inapplicable on less than 0.2 mg. per liter.

When the same reaction is applied in alcoholic solution in the presence of 2:1 sulfuric acid, estrone gives a deep orange-red with green fluorescence, equelin gives a blue, and equelinin an orange pink with 0.3 the

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[38] S. Kober, *Biochem. Z.* **239**, 209-12 (1931); *Biochem. J.* **32**, 357-65 (1938); Saul Louis Cohen and Guy Frederick Marrian, *Ibid.* **28**, 1603-14 (1934); George F. Cartland, Roland K. Meyer, Lloyd C. Miller, and Marshall H. Rutz, *J. Biol. Chem.* **109**, 213-20 (1935); Eleanor H. Venning, Kenneth A. Evelyn, and E. V. Harkness, *Trans. Roy. Soc. Canada* (1936); Kenneth A. Evelyn, *J. Biol. Chem.* **115**, 63-75 (1936); Gregory Pincus, Grace Wheeler, Genevieve Young, and P. A. Zahl, *Ibid.* **116**, 253-66 (1936); Eleanor Hill Venning, Kenneth A. Evelyn, E. V. Harkness, and J. S. L. Browne, *Ibid.* **120**, 225-37 (1937); M. N. Lapiner, V. A. Leontovich, and E. F. Kosheverova, *Problemy Endokrinol.* **2**, No. 1, 27-34 (1937); Carl Bachman, *J. Biol. Chem.* **131**, 455-62 (1939); Wilhelm Dirscherl and Friedrich Zilliken, *Biochem. Z.* **319**, 407-19 (1949).

intensity of estrone at 510 mμ. Thus by correction for the latter two as separately determined, estrone is estimated. Alcoholic types of the keosteroids, the estrogenic diols, act similarly but are separated by trimethylacethydrazine ammonium chloride, Girard's reagent T. Results agree well with infrared spectophotometry. Direct heating of the steroid with sulfuric acid is also applicable.[39]

Guaiacol sulfonic acid,[40] often referred to as thiocol, produces a green color. This is obtained without addition of copper, but copper accelerates it.[41] Ferric chloride produces the same effect, but excess causes fading. The reaction is apparently a catalyzed oxidation, the color being destroyed by peroxide or permanganate. Of related compounds only testosterone oxime, testosterone propionate, and androstenedione give similar absorption curves. Negative results are given by ethynyltestosterone; androsterone; dehydroandrosterone; Δ^5-androstenediol-3-*trans*-17-*cis*; 3,7-androstanedione; 3,11,17-androstanetrione; 11-dehydro-17-hydroxycorticosterone; pregnenin-17-diol-3,17; etioallocholanol-3(β)-17-one; progesterone; cholesterol; estradiol; and estriol.

In the absence of estradiol, equilin, androsterone, testosterone, corticosterone, and cholesterol, the color of 0.005-0.25 mg. with *p*-dimethylbenzaldehyde, *m*-nitrobenzaldehyde, or salicylic aldehyde is suitable.[42] The red color with anhydrous antimony trichloride is also read at 520 mμ,[43] in the absence of estradiol and equelin. Estrone, free from other hormones, reduces phosphomolybdotungstic acid to molybdenum blue under suitable carefully controlled conditions.

Estrone couples with *p*-diazobenzene sulfonic acid to give a red azo dye.[44] Since the reaction depends on the phenolic property, it is given by theelol also. The same basic reaction is obtained by coupling with tetrazotized dianisidine to give a yellow color.[45] The color conforms to Beer's law over the range of 0.01-0.15 mg. per ml. The familiar reaction of phenols with phosphomolybdotungstic acid is applied to estrone,

[39] H. Cohen and R. W. Bates, *J. Clin. Endocrinol.* **7**, 701-7 (1947).

[40] C. M. Szazo and L. T. Samuels, *J. Biol. Chem.* **151**, 587-98 (1943); Harald Oberste-Lehn, *Z. physiol. Chem.* **286**, 1-7 (1950).

[41] Virgil L. Koenig, Francisca Melzer, Clara M. Szazo, and Leo T. Samuels, *J. Biol. Chem.* **141**, 487-93 (1941).

[42] Karl Wulfert, *Acta Chem. Scand.* **1**, 818-32 (1947).

[43] Leonel Fierro del Rio and Delfina Arrieta Aupart, *Rev. inst. salubridad. y enfermedad. trop.* (Mex.) **10**, 167-78 (1949).

[44] Maurice J. Schmulovitz and H. Boyd Wylie, *J. Biol. Chem.* **116**, 415-21 (1936).

[45] Seymour Lieberman, Henry J. Tagnon, and Phyliss Schulman, *J. Clin. Invest.* **31**, 341-5 (1952).

estradiol, and estriol [46] in alkaline solution at 720 mμ. Similar concentrations of the three estrogens give similar color intensities.

Absorption in the ultraviolet is used to determine estrone, estriol, and estradiol.[47] The maximum absorption does not differ enough to permit their differentiation. Therefore, they must be separated quantitatively.[48] A peak at 280 mμ is then suitable for each. After separation they are read above 0.015 mg. per ml. of ethanol. Concentration of urine specimens by freeze-drying is a suitable technic.[49] Urinary phenols must be eliminated.

Appropriate reagents which give no absorption in the ultraviolet are ether freshly distilled from ferrous sulfate, thiophene-free benzene, carbon tetrachloride free from carbon bisulfide, ethanol, disodium acid phosphate, powdered agar USP, granular gelatine, flake graphite, and sulfuric acid. Stop-cock grease is replaced by agar. Hydrochloric acid, filter paper, carbonates, calcium hydroxide, sodium sulfate, toluene, and porcelain boiling stones are eliminated. An exception is the use of hydrochloric acid in the Girard separation where sulfuric acid gives more interfering background material.

Absorption maxima of the benzene sulfonates in the infrared at estrone 10.88μ, equilin 10.96μ, and equilenin 10.45μ [50] permit estimation by a set of 3 simultaneous equations.[51] When heated in ethanol with sulfuric acid for 10 minutes, the fluorescence is read as a measure of estrogens.[52]

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[46] C. Heusghem and M. Vliers, *J. pharm. Belg.* **5**, 276-81 (1950).

[47] Harry B. Friedgood and J. B. Garst, *Recent Progress in Hormone Research* **2**, 31-78 (1948); A. Banchetti, C. Conti, and V. Marescotti, *Folia Endocrinol.* (Pisa) **5**, 161-76 (1952).

[48] Adolf Butenandt and Inge Störmer, *Z. physiol. Chem.* **208**, 129-48 (1932); R. K. Callow, *Biochem. J.* **30**, 906-8 (1936); T. R. Hogness, A. E. Sidwell, Jr., and F. P. Zscheile, *J. Biol. Chem.* **120**, 239-56 (1937); M. V. Mayneord and E. M. F. Roe, *Proc. Roy. Soc. London, Series A* **158**, 634-50 (1937); R. D. H. Heard and M. M. Hoffman, *J. Biol. Chem.* **138**, 651-65 (1941); Samuel R. M. Reynolds and Nathan Ginsburg, *Endocrinology* **31**, 147-61 (1942).

[49] Harry B. Friedgood, Arie J. Haagen-Smit, Josephine B. Garst, and Lotti Steinitz, *Science* **105**, 99-100 (1947).

[50] Jonas Carol, J. C. Molitor, and Edward O. Haenni, *J. Am. Pharm. Assoc., Sci. Ed.* **37**, 173-9 (1948).

[51] Frederick C. Nachod, E. T. Hinkel, Jr., and Calvin Zippin, *Ibid.* **38**, 173-4 (1949).

[52] Gordon A. Groves and Mervyn J. Huston, *Ibid.* **39**, 280-2 (1950); Robert W. Bates and Herman Cohen, *Endocrinology* **47**, 166-92 (1950); C. Hensghen, *J. pharm. Belg.* **7**, 396-402 (1952).

Samples—*Separation of estrogens from phenols volatile with steam.*[53] The solution for use here is that developed on page 000 by the separation of androgens. The scheme is shown in Figure 16. The end product at step 20 is a benzene solution of estrogens in a 500-ml. separatory funnel.

Separation of estriol from estrone and estradiol. The scheme is shown in Figure 17. At step 22 the aqueous phase is transferred to another 500-ml. funnel.

Separation of estrone from estradiol. The scheme is shown in Figure 18. The Girard's reagent T applied here consists of trimethylacethydrazide ammonium chloride.[54] The final solutions resulting at 28, 41, and 48 are suitable for reading in the ultraviolet at 280 mμ or development by other means.

Urine.[55] Acidify 250 ml. of urine to Congo red paper with concentrated hydrochloric acid and extract with four 25-ml. portions of butanol, centrifuging if emulsions form. Wash the combined extracts with 5 ml. of water. Evaporate the solvent *in vacuo*, take up the residue in 2 ml. of ethanol, and add water to make 125 ml.

Evaporate to 100 ml. to boil off the ethanol and acidify the mixture with concentrated hydrochloric acid to Congo red paper. Add an additional 1.5 ml. of acid, close the flask with tinfoil, and autoclave at 120° for 3 hours.

Extract the mixture with four 25-ml. portions of peroxide-free ether (Vol. III, page 79). Wash the combined ether extracts with two 10-ml. portions of water. Evaporate almost to dryness and take up the residue in sufficient hot ethanol to give a total concentration of 0.005-0.03 mg. of hormone per ml. If a precipitate is present, centrifuge. Develop with phenolsulfonic acid. Alternatively, saturate hydrolyzed urine with salt and benzene and extract with benzene.[56]

Procedure—*By phenolsulfonic acid.* Transfer an aliquot of the sample containing 0.005-0.03 mg. of estrone to a tube. Evaporate to dryness at 100° with a current of air filtered through glass wool. Cool

[53] For separation of estrone, estradiol, and estriol by a liquid chromatogram see Benjamin F. Stimmel, *J. Biol. Chem.* **162**, 99-100 (1946).

[54] André Girard and Georges Sandulesco, *Helv. Chim. Acta* **19**, 1095-1107 (1936).

[55] Eleanor Hill Venning, Kenneth A. Evelyn, E. V. Harkness, and J. S. L. Browne, *J. Biol. Chem.* **120**, 225-37 (1937); Cf. R. H. de Girando, *Arch. farm. bioquim. Tucumán* **4**, 261-9 (1949); *Mon. farm. y térap.* (Madrid) **56**, 167-9 (1950).

[56] Carl Bachman and Dorothy Seymour Petit, *J. Biol. Chem.* **138**, 689-704 (1941).

(9) Aqueous potassium hydroxide extract of estrogenic phenols from Fig. 15

(10) Acidify with 1:5 sulfuric acid to Congo red; extract with four 50-ml. portions of ether

(11) Ether phase

Discard aqueous phase

(12) Wash with 5 ml. of distilled water

Wash discarded

(13) Total phenols in ether

(14) Distilled to dryness by steam jacket; taken up in 5 ml. of 95 per cent ethanol; add 20 ml. of distilled water

(15) Total phenols in aqueous ethanol

(16) Steam-distil to 500 ml. of distillate, maintaining volume in funnel at 20 ml., dry by suction with aid of warm bath

(17) Residue of non-steam-volatile phenolic estrogens estrone, estradiol, estriol

(18) Steam-volatile substances

(19) Dissolve residue in 1 ml. of 95 per cent ethanol; add 100 ml. of benzene

(20) Benzene solution of estrogens

FIG. 16. Separation of estrogens from phenols volatile with steam

(20) Benzene solution of estrogens

(21) Extract with three 50-ml. portions of 1.065 per cent disodium phosphate solution

(22) Aqueous phase, "strong" phenol (estriol)

(23) Acidify with 1:5 sulfuric acid to Congo red; extract with four 75-ml. portions of ether

(24) Ether extract

Discard aqueous phase

(25) Wash twice with 5-ml. portions of water

(26) Washed ether extract

Discard washings

(27) Distil off ether; dissolve residue in 5 ml. of 95 per cent ethanol

(28) Estriol fraction

(29) Benzene phase, "weak" phenols (estrone, estradiol)

(30) Reduce volume to 25 ml. by suction with aid of warm water bath; transfer directly to a 250-ml. funnel and dry benzene; rinse in with three 5-ml. portions of 95 per cent ethanol. Evaporate the ethanol

(31) Estrogen residue

(32) Dry the residue in the funnel over phosphorus pentoxide in a vacuum desiccator overnight

(33) Dry residue of estrone-estradiol fraction

FIG. 17. Separation of estriol from estrone-estradiol fraction

(33) Dry residue of estrone-estradiol fraction

(34) Add 2 ml. of glacial acetic acid, then 400 mg. Girard's Reagent T, stopper funnel with an adapter fitted with a calcium chloride tube; heat on a water or glycerol bath at 90-100° for 20 minutes; cool in a desiccator; add 60 ml. of ice-cold water, then 14 ml. of chilled 10 per cent sodium hydroxide solution; mix; extract with four 50-ml. portions of ether

(35) Ether phase, estradiol fraction

(36) Wash with 20 ml. of ice-cold water; add wash to aqueous phase

(37) Washed ether extract

(38) Wash with 20 ml. of 2.5 per cent sodium carbonate solution and three 10-ml. portions of water

(39) Ether extract

Aqueous phase discarded

(40) Distil to dryness; dissolve the residue in 5 ml. of 95 per cent ethanol

(41) Alcoholic solution of estradiol

(42) Aqueous alkaline phase, estrone fraction

(43) Acidify with 12 ml. of concentrated hydrochloric acid; keep at room temperature for 2 hours; extract with three 50-ml. portions of ether in a 250-ml. separatory funnel

Aqueous phase discarded

(44) Ether extract

(45) Wash with 20 ml. of 2.5 per cent sodium carbonate solution and three 10-ml. portions of water

(46) Ether extract

Aqueous phase discarded

(47) Distil to dryness; dissolve the residue in 5 ml. of 95 per cent ethanol

(48) Alcoholic solution of estrone

FIG. 18. Separation of estrone from estradiol

in a desiccator for 1 hour and then add 3 ml. of a sulfuric acid phenol reagent containing 3.6 grams of redistilled phenol to 5.6 grams of concentrated sulfuric acid. Make the additions from a buret to the sample and a blank. Heat at 100° for 20 minutes. Cool at 0° for 5 minutes and add 3 ml. of ice water. Heat at 100° for 3 minutes, cool for 5 minutes, and add 9 ml. of 1:3 sulfuric acid. Read at 420 mμ for inherent color and at 522 mμ for the determination. No fading occurs in an hour, even at as high as 20°. Unless the reading at 522 mμ is at least 6 times that at 420 mμ, a correction for the brown color developed by the reagent with nonestrogens is necessary.[57]

Alternatively correct for interfering colors in the sample.[58] For this purpose add a little acetone just before making to volume for reading. After reading, stopper and let stand in the dark overnight. The color due to estrone fades and the reading after standing is the blank to be subtracted. A modified form of this reagent is applied to determination of estrone in the presence of equelin and equelinin (page 399).

By guaiacolsulfonic acid and copper. Chill a sample containing 0.01-0.04 mg. of test substance in about 0.4 ml. of absolute ethanol, in ice. Add 2 ml. of concentrated sulfuric acid carefully with stirring. Heat in boiling water for 1 minute with stirring and chill again. After 5 minutes add 4 ml. of aqueous 7.5 per cent guaiacolsulfonic acid. Mix and again heat for 2 minutes, stirring at intervals. Chill in ice water and store for 1 hour at 25° in the dark. Add 3.6 ml. of 1:1 sulfuric acid and read after 15 minutes at 635 mμ against a reagent blank.

By tetrazotized dianisidine. As reagent dissolve 25 mg. of dianisidine hydrochloride in 3 ml. of water. Add 0.3 ml. of fresh 5 per cent sodium nitrite solution. After 5 minutes add 0.66 ml. of 5 per cent urea solution. At once add 10.75 ml. of cold 2 per cent sodium carbonate solution. Prepare this reagent anew for each test.

To 0.3 ml. of sample solution containing 0.05-0.15 mg. of test substance add 0.1 ml. of the fresh reagent and mix. Add 8 ml. of 20 per cent sodium chloride solution and 20 ml. of toluene. Shake mechanically for 10 minutes and centrifuge. Read the toluene layer at 425 mμ against a blank.

By fluorescence. Prepare a standard containing 0.01 mg. of estriol in ethanol. Prepare an internal standard by adding 0.01 mg. of estriol

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[57] Benjamin F. Stimmel, *Ibid.* **165**, 73-80 (1946).

[58] M.-F. Jayle, O. Crépy and O. Judas, *Bull. soc. chim. biol.* **25**, 301 S (1943).

to 1 ml. of sample. Use 1 ml. of sample in ethanol without addition. Evaporate to dryness.

To each add 0.5 ml. of ethanol and 5 ml. of 90 per cent sulfuric acid. Shake for 15 seconds and incubate at $80 \pm 5°$ for exactly 10 minutes. Chill with ice and add 15 ml. of 65 per cent sulfuric acid. Mix and read in a fluorometer with a primary filter of 420 mμ and a secondary filter of 525 mμ.

Estradiol, Dihydrotheelin, Dihydrofolliculin, Dihydrostrone, 3,17-Dihydroxy-$\Delta^{1,3,5}$-estratriene

In estradiol the ketone group of estrone has been reduced to an hydroxyl. Thus it has as active groups only phenolics and double bonds. There are both α- and β-forms. After chromatographic separation estradiol, dihydroequelin, and dihydroequelinin are read in the infrared.[59]

The most significant reaction used for its estimation is that with phenolsulfonic acid. In this method the β-form reacts at room temperature but the α-form does not.[60] This furnishes a basis for estimation of each in the presence of the other. The reaction is also given by estrone, estriol, and equilinin when heated, but not cold. The color will determine 5 per cent of either form in the presence of 95 per cent of the other with accuracy usually within 5 per cent. The maximum for the α-isomer is at 523 mμ, for the β-isomer at 528 mμ, and the absorption of the two isomers is the same at 526 mμ. The corresponding reaction with only sulfuric acid present differentiates α- and β-estradiol.[61]

In the absence of estrone, equelin, androsterone, testosterone, corticosterone, and cholesterol, the color of 0.005-0.25 mg. with *p*-dimethylaminobenzaldehyde, *m*-nitrobenzaldehyde, or salicylic aldehyde is suitable for reading.[62] The red color with antimony trichloride is also suitable in the absence of estrone and equelin. Estimation by reading in the ultraviolet is discussed under estrone.

The dried estrogen when heated with 85 per cent phosphoric acid gives a green fluorescence.[63] Intensities are estradiol 2.7, estriol 0.75, desoxycorticosterone acetate 0.3, testosterone 0.1, and progesterone 0.01.

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[59] Jones Carol, *J. Am. Pharm. Assoc.* **39**, 425-32 (1950).

[60] Jones Carol and J. C. Molitor, *Ibid.* **36**, 208-10 (1947).

[61] Ernest J. Umberger and Jack M. Curtis, *J. Biol. Chem.* **178**, 275-87 (1949).

[62] Karl Wulfert, *Acta Chem. Scand.* **1**, 818-32 (1947).

[63] M. Finkelstein, S. Hestrin, and W. Koch, *Proc. Soc. Exptl. Biol. Med.* **64**, 64-71 (1947); M. Finkelstein, *Ibid.* **69**, 181-4 (1948).

There is no fluorescence with cholesterol, dehydrocholic acid, androsterone, androstanediol, pregnandiol, pregnenelone, or allopregnandione.

Procedure—*By phenolsulfonic acid.* Prepare a reagent by slowly adding with mixing 5.6 parts by weight of concentrated sulfuric acid to 3.6 parts by weight of freshly redistilled phenol and cool. The sample should be in solution in ethanol.

To each of two 20×150 mm. test tubes, transfer aliquots of the sample solution containing 0.025-0.1 mg. of total estradiols. Add 1 ml. of α-estradiol standard solution containing 0.1 mg. per ml. to a third test tube and 1 ml. of β-estradiol standard solution containing 0.05 mg. per ml. to a fourth tube. Evaporate all of them to dryness on a steam bath, using a current of air. Dry the tubes in a desiccator over sulfuric acid for 1 hour.

Total estradiols. To one of the sample tubes, the α-estradiol tube, and a blank tube add 3 ml. of reagent from a buret. Stopper with tinfoil-covered corks and heat in boiling water for 20 minutes. Shake vigorously twice during the first 10 minutes to insure thorough mixing with the viscous reagent. After 20 minutes heating, cool in an ice bath for 5 minutes. Leaving the tubes in the ice, add 3 ml. of distilled water from a buret to each tube and mix the contents thoroughly with a glass rod. Place the tubes in boiling water again for exactly 3 minutes. Transfer the tubes to the ice bath for 5 minutes. Dilute the contents of each tube to 15 ml. with 1:3 sulfuric acid and mix well. Read the samples and α-estradiol standard relative to the blank solution at 420 mμ and 526 mμ.

$$\text{Total estradiol in mg.} = \frac{E_{526\text{-m}\mu\text{ sample}} - E_{\frac{420\text{-m}\mu\text{ sample}}{2}}}{E_{526\text{-m}\mu\text{ standard}} - E_{\frac{420\text{-m}\mu\text{ standard}}{2}}} \times 0.1$$

β-Estradiol. To the other sample tube, the β-estradiol standard tube, and a blank tube, add 3 ml. of the reagent from a buret. Mix each tube thoroughly with a glass rod. Let the tubes stand at room temperature for 20 minutes with stirring every 5 minutes. At the end of 20 minutes cool in an ice bath for 5 minutes and dilute each to 15 ml. with 1:3 sulfuric acid. Read at 420 mμ and 528 mμ.

$$\beta\text{-Estradiol in mg.} = \frac{E_{528\text{-m}\mu\text{ sample}} - E_{\frac{420\text{-m}\mu\text{ sample}}{2}}}{E_{528\text{-m}\mu\text{ standard}} - E_{\frac{420\text{-m}\mu\text{ standard}}{2}}} \times 0.05$$

α-Estradiol = total estradiols − β-estradiol.

By sulfuric acid. Evaporate two samples containing 0.005-0.05 mg. of estradiols to dryness.

β-Estradiol. To one sample add 5 ml. of 30 per cent sulfuric acid-20 per cent butanol and heat for 6 minutes at 100° Read at 524 mμ. The color is stable for 1 hour, and there is no interference by 0.05-0.1 mg. of estradiol, estrone, equilin, α-dihydroequilin, equilenin, and estriol.

Total estradiols. Repeat the operation with 73.5 per cent sulfuric acid, heating for 12 minutes, and read at 455 mμ.

α-Estradiol. Determine by difference.

By fluorescence. Mix 1 ml. of methanol solution of estrone or estradiol containing not over 0.00025 mg. with 8 ml. of 60 per cent sulfuric acid. Heat in a boiling water bath for 5 minutes and cool. Read the fluorescence.[64] Estriol gives about 5 per cent as much fluorescence as estrone and estradiol. That due to other steroids and cholesterol is not significant.

Theelol, Estriol, 3,16,17-Trihydroxy-$\Delta^{1,3,5}$-estratriene

Estriol contains one more phenolic group than estradiol and reacts very similarly. The separations are shown under estradiol (Figs. 17-18). When 1 mg. or more per liter is developed with phenolsulfonic acid (page 408), accuracy is better than ±10 per cent.[65] Estriol is read in the ultraviolet at 280 mμ as discussed under estrone (page 401).

Equelin, 3-Hydroxy-17-keto-$\Delta^{1,3,5,7}$-estratetraene

Equelin can be very simply described as estrone with an additional unsaturation. It follows that it can be expected to give the corresponding ketonic reactions. The hydroxyl groups are also a point of attack. Thus, the color of 0.005-0.25 mg. of equelin with *p*-dimethylaminobenzaldehyde, *m*-nitrobenzaldehyde, or salicylic aldehyde is suitable for reading in the absence of estrone, estradiol, androsterone, testosterone, corticosterone, and cholesterol.[66] The red color with antimony chloride is also suitable in the absence of estrone and estradiol (page 394).

Equelin and equilinin give stable colors with dibromoquinonechloroimide, whereas estrone does not. The interference of equelin and equelinin is resolved by conversion to benzene sulfonates by reaction

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[64] Joseph W. Jailer, *Endocrinology* **41**, 198-201 (1947).
[65] Cf. Hellmuth Winkler, *Klin. Wochschr.* **21**, 1080-1 (1942).
[66] Karl Wulfert, *Acta Chem. Scand.* **1**, 818-32 (1947).

with benzenesulfonylchloride. Then only equelin gives a reaction with dibromoquinonechloroimide.[67]

Equeleinin is a β-naphthol with the α-position unsubstituted and yields a red color with dibromoquinonechloroimide which is extractable with chloroform. The extract must be alkali-washed to remove brown by-products. The maximum intensity is attained within an hour and conforms to Beer's law over the range 520-540 mμ. Equelin slowly develops about half the intensity under the same conditions. Estrone gives practically no color.

Procedure—*Reaction with dibromoquinonechloroimide. After esterification.* Carefully evaporate a sample containing 0.6-0.8 mg. of mixed estrogenic ketosteroids just to dryness with a stream of air. Cool in a vacuum and add 2 ml. of dry pyridine and 0.2 ml. of benzene sulfonyl chloride. Stopper and let stand overnight. Transfer to a separatory funnel with 15-20 ml. of water and rinse in with 10 ml. more. Extract by shaking with about 20 ml. of chloroform for at least 1 minute. Remove the chloroform layer and again extract with about 20 ml. Evaporate the combined extracts to dryness and take up in 2 ml. of ethanol.

As a buffer for pH 5.2-5.4 dissolve 22 grams of sodium acetate trihydrate or 13.3 grams of anhydrous salt in about 60 ml. of water, add 20 ml. of glacial acetic acid, and dilute to 100 ml. Add 4 ml. of this buffer. Add 2 ml. of 0.5 per cent solution of dibromoquinonechloroimide in ethanol. An immediate and rapid development of red color indicates incomplete esterification due to interference by moisture. After 4 hours for reaction add 7 ml. of chloroform and mix. Add 20 ml. of 10 per cent sodium hydroxide solution and shake for 1 minute. Remove the chloroform extract, filter through paper, and read at 530 mμ against a reagent blank.

Without esterification. Transfer a solution containing 0.3-0.5 mg. of mixed estrogenic ketosteroids to a separatory funnel and add 5 ml. of the buffer for pH 5.2-5.4. Add 1 ml. of 0.5 per cent reagent and let stand for 2 hours. Add 7 ml. of chloroform and mix. Extract by shaking for 1 minute with 20 ml. of 10 per cent sodium hydroxide solution. Remove the clear chloroform layer, filter through paper, and read against a reagent blank at 530 mμ.

Estrone in the mixture. As reagent dissolve 1.054 gram of ferrous ammonium sulfate in about 20 ml. of water and add 1 ml. of 30 per

[67] Daniel Banes, *J. Am. Pharm. Assn.* **39**, 37-41 (1950).

cent hydrogen peroxide. Mix, heat until effervescence ceases, and dilute to exactly 50 ml. Dilute 3 ml. of this volumetrically to 100 ml. with concentrated sulfuric acid. Add to redistilled crystallized phenol 1.13 times its weight of the iron-sulfuric acid solution and mix occasionally until liquefied. Shake until homogeneous and let stand for 16-24 hours in the dark. Add to the weighed mixture 23.5 per cent of its weight of a mixture of 100 volumes of concentrated sulfuric acid and 110 volumes of water. Shake until homogeneous and store, well stoppered, in the dark. For use dilute 10 ml. of this stock to 100 ml. with 2:1 sulfuric acid just prior to use, shaking until homogenous.

To 1 ml. of ethanol containing 0.06-0.08 mg. of mixed estrogenic ketosteroids add 10 ml. of the reagent. Heat at 75-80° for 2 hours. Cool in water and read against a blank at 510 mμ. From a standard curve calculate the approximate equelin content from the result obtained by esterification.

Calculate equelinin from the result without esterification after correcting for the color due to equelin as previously calculated.

Calculate estrone from the value obtained at 510 mμ, applying corrections for equelin and equelinin from standards similarly treated and read.

Recalculate equelin with corrections now available for equelinin and estrone. Apply this recalculation to equelin and estrone.

Dihydroequelenin

This hormone gives the general ketone and phenol reactions of estrone. By coupling with diazotized *p*-nitrobenzeneazodimethoxyaniline, amounts of dihydroequilenin and equilenin as low as 0.01 mg. per ml. are determined with accuracy to ±10 per cent.[68]

Equilenin, 3-Hydroxy-17-keto-$\Delta^{1,3,5:10,8,6}$-estrapentaene

Equelinin is estrone with three more double bonds. It follows that it can give ketone or phenol reactions. Its determination in the presence of equelin is described under the latter.

Corticosterone, 17(β)-[1-keto-2-hydroxymethyl]-Δ^4-androsterone-3-one-1(β)-ol, 11-21-Dihydroxyprogesterone

It follows from the structural name that corticosterone will give the hydroxyl and ketone reactions of other hormones. Thus the color de-

[68] Walter Marx and Harry Sobotka, *J. Biol. Chem.* 124, 693-8 (1938).

veloped from 0.005-0.25 mg. of corticosterone with *p*-dimethylaminobenzaldehyde, *m*-nitrobenzaldehyde, or salicylic aldehyde may be read in the absence of estrone, estradiol, equelin, androsterone, or cholesterol.[69]

Another method of estimation is by the reducing value in glacial acetic acid on phosphomolybdic acid to give molybdenum blue.[70] Beer's Law also holds for desoxycorticosterone and 21-hydroxypregnenolone by this method. The arsenomolybdate reagent [71] reacts with all steroids containing a primary or secondary γ-ketol function, an α-β-unsaturated 3-ketone group, or both. Accuracy is to ± 2 per cent. The determination of cortisone as the blue deformazan (page 414) is applicable to corticosterone.

Procedure—To 0.1 ml. of sample solution containing 0.001-0.01 mg. of the steroid add 2 ml. of phosphomolybdic acid reagent (Vol. III, page 205). Mix and heat in boiling water for exactly 60 minutes. Cool and read within 5 minutes at 650-660 mμ.

11-Dehydrocorticosterone

The reaction of cortisone to form a blue diformazan (page 414) is also applicable to this compound.

17-Hydroxycorticosterone

This hormone is determined by oxidation of its primary α-ketol group to liberate formaldehyde. Cortisone gives the same reaction and the method is shown (page 415) under that topic.

Desoxycorticosterone, 21-Hydroxyprogesterone, 17(α)-[1-keto-2-hydroxylethyl]-Δ^4-Androstene-3-one

Desoxycorticosterone is estimated by the color with alcoholic sulfuric acid following the procedure for testosterone (page 397) in the absence of testosterone, Δ^4-androsterone-3β-17-dione, and dehydroandrosterone. The determination of cortisone as the blue diformazan (page 414) is applicable to 11-desoxycorticosterone. Corticoids in general are determined with chromotropic acid.

Procedure—*Urine.* Reflux 50 ml. of sample with 7.5 ml. of concentrated hydrochloric acid for one-half hour and cool. Extract with 50,

[69] Karl Wulfért, *Acta Chem. Scand.* **1**, 818-32 (1947).
[70] R. D. H. Heard and H. Sobel, *J. Biol. Chem.* **165**, 687-98 (1946).
[71] W. Raab, *Endocrinology* **28**, 325-36 (1941).

25, and 25 ml. of chloroform. Wash the combined extracts with 25 ml. of 8 per cent sodium hydroxide solution and then with 25 ml. of water. Evaporate the washed chloroform extract to dryness and take up in 2 ml. of sirupy phosphoric acid by heating. Add 8 ml. of water and 4 ml. of 0.23 per cent solution of potassium iodate in 2:45 sulfuric acid. After 15 minutes add 1.5 ml. of 6 per cent stannous chloride in 1:10 hydrochloric acid. Transfer to a flask and distil, passing a slow stream of moisture-saturated air as a carrier. Collect nearly 20 ml. and dilute to that volume. Mix 5 ml. of the diluted distillate with 4 ml. of concentrated sulfuric acid and add 0.2 ml. of 5 per cent aqueous solution of chromotropic acid. Heat in boiling water for 30 minutes, cool, and read at 550 mμ. Up to 0.25 mg. the results are linear against desoxycorticosterone acetate.

17-Hydroxy-11-desoxycorticosterone

The diformazan method of determining cortisone is also applicable here.

Cortisone, 11-Dehydro-17-hydroxycorticosterone

The cortisone of commerce is the ester, 11-dehydro-17-hydroxycorticosterone-21-acetate. The reaction of cortisone with dianisole-bis-4,4'-(3,4-diphenyl)tetrazolium chloride in a solution made alkaline with tetramethyl ammonium hydroxide is appropriate for reading.[72] The same reaction is given by 2,3,5-triphenyl tetrazolium chloride but with half the intensity.

These reactions are like that of the α-ketol group of fructose to produce deep red water-insoluble formazans[73] or blue diformazans.[74] Therefore, reducing sugars must be absent. Extraction with absolute ethanol or isopropanol eliminates them.

So far as steroids are concerned, the reaction is specific for a ketone group on carbon 11 and a hydroxyl group on carbon 17. Thus the reaction is also given by corticosterone, 11-desoxycorticosterone, 17-hydroxy-11-desoxycorticosterone, and 11-dehydrocorticosterone. It is expected that 17-hydroxycorticosterone, dihydrocorticosterone, 6-dehydrocorticosterone, and 21-acetoxypregnenolone will also so react. The

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[72] W. J. Mader and R. R. Buck, *Anal. Chem.* **24**, 666-7 (1952).

[73] R. Kuhn and D. Jerchel, *Ber.* **74B**, 941-8 (1941).

[74] A. M. Rutenberg, R. Gofstein, and A. Seligman, *Cancer Research* **10**, 113-21 (1950).

method as reported will determine 0.01-0.17 mg. per ml. Paper chromatography extends the range.

The primary α-ketol group in cortisone leads to quantitative liberation of formaldehyde on treatment with periodic acid.[75] The solution is treated with sodium sulfite, the iodide precipitated with silver sulfate, the precipitated silver iodide separated, and the solution used directly. Direct distillation of the formaldehyde gives about 80 per cent recovery, but this is increased to 95 per cent by use of a Conway microdiffusion unit.[76] The same reaction is given by 17-hydroxycorticosterone. They are also referred to as *formaldehydogenic steroids.*

Cortisone reacts with anthrone in 96 per cent sulfuric acid to give peaks in the absorption curve at 415, 480, 590, and 635 mμ.[77] That at 480 mμ is preferable for reading. After heating with organic or inorganic alkali, cortisone acetate shows an absorption maximum at 373 mu.[78] Heating after the maximum is developed does not alter it. Progesterone and testosterone interfere.

Sample—*Tablets.* Grind and extract with absolute ethanol or isopropanol. Dilute to contain about 0.12 mg. of cortisone acetate per 10 ml.

Ointments. Suspend in hot absolute ethanol for extraction. Decant the extract and dilute to about 0.12 mg. of cortisone acetate per 10 ml.

Procedure—*As a deformazan.* To 10 ml. of sample solution in absolute ethanol add 1 ml. of a reagent prepared by 1:9 dilution of 10 per cent aqueous tetramethyl ammonium hydroxide with ethanol. Add 1 ml. of 0.5 per cent solution of dianisole-bis-4,4′-(3,5-diphenyl)-tetrazolium chloride in ethanol, prepared fresh daily. After 15-20 minutes, read at 510 mμ against a reagent blank. The color is stable for at least an hour.

By heating in alkaline solution. Dilute a solution of sample in methanol to 0.02-0.08 mg. per ml. Evaporate 1 ml. to dryness and dissolve the residue in 5 ml. of 10 per cent solution of tetraethyl ammonium hydroxide. Heat at 70° for 35 minutes. Cool and read at 373 mμ against a reagent blank.

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[75] J. Rabinovitch, J. Decombe, and A. Freedman, *Lancet* 261, 1201-2 (1951).

[76] A. A. Henly and Marjorie Potter, *Lancet* 262, 697-8 (1952).

[77] Morris M. Graff, John T. McElroy and Albert L. Mooney, *J. Biol. Chem.* 195, 351-6 (1952); Joseph J. Holechek and Annie R. Collins, *Anal. Chem.* 25, 991-3 (1953).

[78] John M. Cross, Henry Eisen, and Richard D. Kedersha, *Anal. Chem.* 24, 1049-50 (1952).

In urine as formaldehyde. The sample should be preserved with sodium sulfite and acetic acid. Heat a 50-ml. portion for 15 minutes in a closed flask at 60°, with 0.12 ml. of concentrated sulfuric acid and 60 mg. of sodium sulfite. Let cool to room temperature and add 25 per cent sodium hydroxide solution to pH 6-7.

Extract with 3 sucessive 7.5-ml. portions of chloroform. Combine the extracts and centrifuge to destroy the emulsion. Dry over sodium sulfate and remove the chloroform under vacuum at 40°. Dissolve the residue in 1.5 ml. of benzene and transfer to a separatory funnel. Complete the transfer with another 1.5 ml. of benzene used in small portions.

Extract the cortisone from the benzene with 4 successive 5-ml. portions of water and combine them. Transfer the cortisone to chloroform by 3 successive extractions with 4-ml. portions of chloroform. Evaporate the combined chloroform extracts to dryness under vacuum at 40°. To the residue add 1 ml. of 0.23 per cent potassium periodate solution in 2:45 sulfuric acid. Let stand for 45 minutes and add 0.5 ml. of 4 per cent sodium sulfite solution. Precipitate the iodide in the oxidation mixture by addition of 0.5 ml. of 0.3 per cent silver sulfate solution. Filter through sintered glass and use the filtrate as sample.

To 0.6 ml. of sample solution add 1 ml. of 5 per cent aqueous chromotropic acid and mix. Place in boiling water for 30 minutes, out of the light. Keep steam away from the mouth of the tube. Cool and make up to 2 ml. with 1:3 sulfuric acid. Mix and read at 550 mμ against a reagent blank.

By anthrone. Dilute a 0.5-1.5 mg. sample in absolute ethanol to 2 ml. with the same solvent. Run in 2 ml. of 0.2 per cent solution of anthrone in 96 per cent sulfuric acid in such a way as to form a layer under the sample. Mix with a rod having a flattened end, slowly enough to avoid boiling off the ethanol. Read after 1 hour at 480 mμ against a reagent blank.

PROGESTERONE, 17(α or β)-[1-KETOETHYL]-Δ^4-ANDROSTEN-3-ONE

The ketonic and hydroxyl functions furnish the reactive groups of this compound. Thus in the absence of testosterone, androsterone, and dehydrocholic acid it gives a red with 3,5-dinitrobenzoic acid in aqueous benzyl trimethyl ammonium hydroxide [79] (page 394).

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[79] Robert P. Tansey and John M. Cross, *J. Am. Pharm. Assn.* 39, 660-3 (1950).

Pregnenolone

Read pregnenolone and its acetate in acetone at 935, 1047, or 1708 mμ, all of which are nonspecific bands.[80]

Pregnanediol, Pregnane-3(α),20(α)-diol

Pregnanediol is estimated by the reaction with phenolsulfonic acid. Color is also developed by reaction with concentrated sulfuric acid,[81] provided neither cholesterol nor neutral 17-ketosteroids are present.

Pregnanediol gives a stable, quantitative color reaction with acetyl chloride and zinc chloride.[82] The color development is affected by all the usual factors, concentration of reagents and test substance, temperature, and time. Unlike many methods, it is not feasible to read at a stable maximum with the usual size of samples. It is accurate to within 4 per cent. Synthetic 3β, 20β-allopregnanediol prepared from pregnenolone is a suitable standard.[83]

Sample—*Urine*.[84] Heat 100 ml. of urine to boiling under a reflux with 50 ml. of sulfur-free toluene. Add 10 ml. of concentrated hydrochloric acid through the condenser and boil for exactly 15 minutes. Cool and separate the layers. Filter the toluene and emulsion with suction. Wash the toluene filtrate with two 15-ml. portions of 0.4 per cent sodium hydroxide solution, then with similar volumes of water. Boil until water is evaporated as indicated by smooth boiling. Add 10 ml. of 2 per cent solution of sodium hydroxide in absolute methanol. Evaporate until a granular precipitate separates. Filter through fritted glass to give a yellow or green filtrate. Wash the residue with 15 ml. of hot toluene, evaporate the toluene nearly to dryness on a hot plate, and finish *in vacuo* in boiling water. Take up the residue in 5 ml. of acetone. Add 20 ml. of 0.4 per cent sodium hydroxide solution and boil for 3 minutes. Chill in a refrigerator for an hour.

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80 G. Papineau-Couture and R. A. Burley, *Ibid.* **39**, 683-6 (1950).

81 Henry S. Guterman, *J. Clin. Endocrin.* **4**, 262-7 (1949); **5**, 407-11 (1945).

82 Joseph W. Golzieher, *J. Lab. Clin. Med.* **33**, 251-3 (1948).

83 Ruth M. Haslam and W. Klyne, *Lancet* **262**, 399 (1952).

84 I. F. Sommerville, G. F. Marrian, and R. J. Kellar, *Ibid.* **255**, 89-90 (1948); Henry S. Guterman and Madeline S. Schroeder, *J. Lab. Clin. Med.* **33**, 356-66 (1948); Cf. I. F. Sommerville, Nancy Gough, and G. F. Marrian, *J. Endocrinol.* **5**, 247-57 (1948); Cf. D. Huber, Borth de Watteville, and R. Bethoux, *Ann. biol. clin.* (Paris) **8**, 518-19 (1950); Sergio Romo L., *Tesis. quim., Univ. Chile*, **2**, 110-21 (1951); Hedwig Ehrlich-Gonolka and Fritz Cekon, *Wien. med. Wochschr.* **101**, 434-5 (1951); T. Dati, G. De Angelis and A. Borgia, *Ricera sci.* **21**, 1791-7 (1951).

Filter on an inorganic filter, discard the filtrate, and wash the precipitate with 15 ml. of water. Take up the precipitate with 10 ml. of boiling absolute ethanol. Add 1-2 mg. of activated carbon, heat in boiling water for 2 minutes, filter, and evaporate the filtrate to dryness for development with sulfuric acid.

Alternatively,[85] adjust the pH of 250 ml. of urine to exactly 10 by dropwise addition of 20 per cent sodium hydroxide solution. Extract with exactly 150 ml. of butanol. Separate the extract and develop the color with phenolsulfonic acid as described for estrone, (page 000). Correct for incomplete extraction by multiplying the result by 1.18.

Procedure—*By sulfuric acid.* The sample should be available as a dried residue containing not over 0.5 mg. of pregnanediol. Add 10 ml. of concentrated sulfuric acid and store at 25° for 1 hour, shaking occasionally. Read at 430 mμ.[86]

By acetyl chloride and zinc chloride. Pipet an aliquot of sample in ether-alcohol to contain 0.1-1 mg. of pregnanediol into a 10-ml. glass-stoppered volumetric flask, and evaporate the solvent. Add 6 ml. of 38 per cent zinc chloride in glacial acetic acid and 2.5 ml. of acetyl chloride. At the same time treat a standard containing 0.5 mg. of pregnanediol in the same way. Incubate the flasks at 50° for 30 minutes and cool in ice water. Allow to stand at room temperature for 20 minutes and then adjust the volume with the zinc chloride reagent. Mix carefully and read in the range of 370-450 mμ. Photometric comparison with a standard corrects for many variations in the degree of development of the color.

Stilbestrol, 4,4'-Dihydroxystilbene

The normal stilbestrol is the *trans* form. In common with many stilbene derivatives, the *cis* form is known as the iso-form. The conventional reduction of molybdate to molybdenum blue is used for colorimetric estimation of stilbestrol.[87] The reaction follows Beer's law for aliquots containing up to 0.1 mg. The reaction with phosphotungstic-

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85 M. F. Jayle, O. Crépy, and P. Wolf, *Bull. soc. chim. biol.* **25**, 308-17 (1943).

86 J. M. Bedolla and M. L. Cegama, *Rev. Arch. med. Exptl.* (Madrid) **12**, 151-9 (1949).

87 J. Cheymol and A. Carayon-Gentil, *Bull. soc. chim. biol.* **27**, 376-82 (1945); A. Carayon-Gentil and J. Cheymol, *Ann. pharm. franç.* **6**, 129-36 (1948); F. H. Malpress, *Biochem. J.* **43**, 132-6 (1948).

phosphomolybdic acid described for diethylstilbestrol is also applicable,[88] although the color fades undesirably rapidly.

A reaction product of stilbestrol with excess bromine in acetic acid at 100° for 1 minute forms a violet colloidal dispersion with ethanol and water. When extracted into chloroform for reading it is orange-red and fades rapidly.[89] The reaction is not given by stilbestrol diacetate or dipropionate, or by hexestrol. Therefore, they must be saponified for estimation. Dienestrol, sucrose, and many aldehydes interfere. Stilbestrol is stable but dienestrol and isodienestrol are rapidly oxidized by the atmosphere.[90] Stilbestrol also combines with diazotized sulfanilic acid [91] and can be estimated by nitration.

Sample—*Tablets.* Shake a sample of finely powdered tablets containing about 2 mg. of stilbestrol with 25 ml. and 25 ml. of ethyl acetate for 1 hour. Filter, wash the residue with water, and evaporate the filtrate. Take up in absolute methanol and refilter as the sample. Direct extraction with methanol gives a turbid solution and the solubility of propionates is too low.

Oil. Reflux a sample containing about 2 mg. of stilbestrol with 15 ml. of 20 per cent potassium hydroxide in methanol. After saponification is complete, dilute with about 60 ml. of water and extract the unsaponifiables with petroleum ether. Acidify the aqueous layer with 1:1 hydrochloric acid and extract with 20 ml. and 20 ml. of ether. Wash the combined extracts with 1 per cent sodium bicarbonate solution, then with water. Evaporate the ether extract, take up in methanol, and dilute to 50 ml. with the same solvent. The result includes both free and esterified stilbestrol.

Urine. Filter urine, made acid to Congo red with concentrated hydrochloric acid, and extract a sample containing 0.5-2 mg. of stilbestrol with 50 ml. and 50 ml. of ether. Shake the combined extracts with 25 ml. and 25 ml. of saturated sodium bicarbonate solution to remove glucronides. Save this extract for separate treatment.

Extract the stilbestrol from the ether with 25 ml. and 25 ml. of 8

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88 J. Cheymol and A. Carayon-Gentil, *Bull. soc. chim. biol.* **28**, 723-9 (1946); R. A. Dunford, *Can. J. Research* **27B**, 646-52 (1949).

89 T. Tusting Cocking, *Analyst* **68**, 144-6 (1943).

90 F. L. Warren, F. Goulden, and Alice M. Robinson, *Biochem. J.* **42**, 151-6 (1948).

91 Ernst Huf and Grete Widmann, *Z. physiol. Chem.* **274**, 88-95 (1942); *Ibid* **279**, 119 (1943); Gino Carrara, *Ibid.* **279**, 117-19 (1943).

per cent sodium hydroxide solution. Acidify the combined alkaline extracts with 12.5 ml. of concentrated hydrochloric acid. Extract the stilbestrol from this acid solution with 25 ml. and 25 ml. of ether. Wash the combined ether extracts with 25 ml. of saturated sodium bicarbonate solution and then with 25 ml. of water. Dry the ether solution with anhydrous sodium sulfate. Pass this dry solution through a column of aluminum oxide and wash with 5 ml. of dry ether. Add 2 grams of calcium hydroxide to the ether solution. Stir, let settle, and decant. Wash the calcium hydroxide with ether and decant. Evaporate ether from the calcium hydroxide precipitate, suspend it in 25 ml. of water, and dissolve with 7.5 ml. of concentrated hydrochloric acid. Extract this acid solution with 25 ml. and 25 ml. of ether. Wash the ether extract with water and dry with anhydrous sodium sulfate. Evaporate the dried ether extract to dryness and take up in 6 ml. of glacial acetic acid for estimation of stilbestrol by nitration.

Urine glucuronides. They were recovered in sodium bicarbonate extracts. Add 1:1 hydrochloric acid until acid to Congo red. Extract with 25 ml. and 25 ml. of ether. Extract the combined ether extracts with 25 ml. and 25 ml. of saturated sodium bicarbonate solution. Add 1:1 hydrochloric acid to make these sodium bicarbonate extracts acid to Congo red. Extract with 25 ml. and 25 ml. of ether. Wash the ether extract with 25 ml. of water and dry with anhydrous sodium sulfate. Again extract with 25 ml. and 25 ml. of saturated sodium bicarbonate solution. Neutralize to Congo red with 1:1 hydrochloric acid and add 17 ml. of concentrated hydrochloric acid. Reflux for 30 minutes and cool. Complete as for stilbestrol from "Extract the stibestrol from this acid solution . . . ."

Procedure—*By ammonium molybdate.* Evaporate an aliquot of the sample containing not over 0.1 mg. of stilbestrol to dryness *in vacuo.* Add 2 ml. of a 0.5 per cent solution of ammonium molybdate in 1:1 sulfuric acid. Shake vigorously and heat for 3 minutes in boiling water with agitation. Cool in ice water for 1 minute and dilute to volume with water. Cool, stopper, and mix by inversion for 30 seconds. Read at 660 mμ against a sample blank in which the ammonium molybdate solution was replaced by 2 ml. of 1:1 sulfuric acid.

By diazotized sulfanilic acid. As coupling agent dissolve 4.5 grams sulfanilic acid in 60 ml. of concentrated hydrochloric acid. Chill 1.5 ml. of this and add 1.5 ml. of 5 per cent sodium nitrite. After 5 minutes add 5 ml. more of 5 per cent sodium nitrite solution. Dilute after 5

minutes to 50 ml. As buffer for pH 12 dissolve 20 grams of boric acid in 280 ml. of 4 per cent sodium hydroxide solution and dilute to 1 liter.

Add 1 ml. of sample containing 0.05-0.25 mg. of stilebstrol in methanol to 8 ml. of the buffer. Heat at 80-85° for 5 minutes. Cool to 20° and add 2 ml. of the cold coupling agent and a drop of saturated aqueous calcium chloride. Mix, filter, and read at 500 mμ against a reagent blank.

By nitration. See page 423.

DIETHYLSTILBESTROL, α,α'-DIETHYL-4,4'-DIHYDROXYSTILBENE

Diethylstilbestrol is typical of a family of phenolic synthetic hormones which are determinable by characteristic reactions. In the absence of other phenolic reducing substances, diethylstilbestrol is estimated by reduction of the labile complex phosphomolybdic-phosphotungstic acids by the two phenolic hydroxyls to give a collodial blue dispersion of tungstic oxides.[92] The color is stable and develops fully within an hour. Alcohol, sugars, talc, fatty acids, and soaps do not interfere in the reaction. Water-soluble colors are eliminated by extraction of the sample.

The familiar xanthoproteic acid reaction is applicable in the absence of other phenol compounds.[93] The yellow color developed is stable and not greatly affected by variation in concentration of reagents. The accuracy is usually to 1 per cent. The nitroso compound in alkaline solution is also read.[94]

Another method calls for reaction in chloroform with antimony pentachloride[95] to give a red color. The color is given also by diethylstilbestrol glycuronide and dienestrol. Practically the glucuronide is hydrolyzed for determination. Phthalein indicators, phenolic bodies, and lipides should be absent. Estrone and estradiol do not interfere. Phosphoric acid is less destructive to the steroids than hydrochloric or sulfuric acid.

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92 Manuel Tubis and Albert Bloom, *Ind. Eng. Chem., Anal. Ed.* 14, 309 (1942).

93 Earl B. Dechene, *J. Am. Pharm. Assn.* 30, 208-9 (1941); F. H. Malpress, *Biochem. J.* 39, 95-8 (1945); A. Mariani and L. Tentori, *Ann. chim. applicata* 39, 222-6 (1949).

94 A. Mariani and L. Tentori, *Rend. ist. super. sanita.* (Rome) 12, 304-10 (1949).

95 E. Dingemanse, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.* 10, 118-22 (1940); *Nature* 145, 825 (1940); E. Dingemanse and R. Tyslowitz, *Endocrinology* 28, 450-7 (1941); A. D. Bass and W. T. Salter, *Yale J. Biol. Med.* 15, 729-33 (1943); R. S. Teague and Albert E. Brown, *J. Biol. Chem.* 189, 343-54 (1951).

Samples—*Compressed, colorless tablets.* Weigh several tablets equivalent to 10 mg. of diethylstilbestrol and grind to a powder. Accurately weigh a sample equivalent to approximately 5 mg. of diethylstilbestrol, add 30 ml. of ethanol, and reflux for 15 minutes on a water bath. Cool the solution, dilute to 100 ml. mix, and filter. Develop with either phosphotungstic-phosphomolybdic acid or nitric acid.

Colored tablets. Prepare a powdered sample as above and disperse in 50 ml. of water. Extract with one 20-ml. and three 10-ml. portions of ether. Combine the ether extracts and evaporate. Dissolve the residue in 30 ml. of ethanol, dilute to 100 ml. with water, and filter. Develop with phosphotungstic-phosphomolybdic acid or nitric acid.

Oils. Dissolve a sample containing about 5 mg. of diethylstilbestrol in sufficient petroleum ether to give a limpid liquid. Extract successively with 15, 10, and 10 ml. of 4 per cent sodium hydroxide solution. Acidify the combined extracts with 1:9 sulfuric acid and extract successively with 20, 15, and 10 ml. of ether. Wash the combined extracts with water and dilute to a known volume for aliquoting. Develop with nitric acid.

Urine. Content under 1 mg. per liter. Make a portion of urine acid with 1:1 phosphoric acid, cool, and extract thrice with twice the volume of peroxide-free ether. Wash the ether with 5 per cent by volume of 1:1500 phosphoric acid. Wash this aqueous extract twice with double the volume of ether. Concentrate the ether solutions and extract with half the volume of 9 per cent sodium bicarbonate, then with two quarter volumes of the same strength, finally with two quarter volumes of water. Wash the combined aqueous extracts with 20 per cent of its volume of ether. Wash this ether extract twice with quarter-volume portions of 9 per cent sodium bicarbonate solution and once with an equal portion of water. The combined aqueous solutions now contain the conjugated diethylstilbestrol and the combined ether solutions the free diethylstilbestrol.

Free diethylstilbestrol. Wash the ether solution with one-tenth volume of fresh 10 per cent sodium carbonate solution. Backwash the aqueous extract with 2 volumes of ether. Discard the aqueous washings and pool the ether layers.

Wash the ether layer with one-tenth volume of 1:150 phosphoric acid. Backwash that washing with 2 volumes of ether, add the ether to the ether solution, and discard the aqueous layer. Wash the ether layer

twice with one-tenth volume of water. Backwash those washings with 2 volumes of ether, add the ether to the ether layer, and discard the washings.

Evaporate the ether layer almost to dryness, add 1 ml. of ethanol, and take to dryness under nitrogen. Take up in ethanol, dilute to 25 ml. and store in a refrigerator until ready to use. The solution should be no more than faintly yellow. Develop by antimony pentachloride.

If necessary to prevent discoloration with antimony pentachloride reagent, purify as follows: Dilute the solution in ethanol with 5 volumes of ether. Wash with one-tenth volume of 0.04 per cent sodium hydroxide solution. Backwash the washings with 2 volumes of ether, add the ether to the ether solution, and discard the washings. Extract the combined ether layers twice with half volumes of 4 per cent sodium hydroxide solution, and thrice with 0.2 volumes. Combine those aqueous extracts and wash with 0.3 volume of ether. Backwash the ether twice with 20 per cent of its volume of 4 per cent sodium hydroxide solution. Discard the ether and combine the alkaline solutions of diethylstilbestrol. Warm to drive off the dissolved ether and cool. Adjust to pH 11 with 1:1 phosphoric acid, using a glass electrode. Extract successively with two half volumes of ether and thrice with quarter volumes. Combine the ether layers, discard the aqueous layer, and revert to "Wash the ether layer with one-tenth volume of 1:150 phosphoric acid." Carry it through ". . . no more than faintly yellow."

Conjugated diethylstilbestrol. Make the bicarbonate solution acid to Congo red paper with 1:1 phosphoric acid. Warm and stir to drive off carbon dioxide. Cool and extract thrice with double the volume of ether. Wash the combined ether extracts with 5 per cent of its volume of 1:1500 phosphoric acid. Backwash these washings with 2 volumes of ether, combine the ether extracts, and discard the aqueous layer. Evaporate the ether extracts to dryness, the last part under nitrogen, take up the residue in 15 ml. of hot 0.4 per cent sodium hydroxide solution, using additional alkaline solution for rinsing. Cool and add 1:15 phosphoric acid until the pH is 3.45 by a glass electrode. Dilute to a known volume and take 20-ml. aliquots. Autoclave for 90 minutes at 180°, cool, and transfer to a separatory funnel with 20 and 10 ml. of water. Wash with 1 volume of ether, twice with half volumes, once with 0.2 volume. The combined ether solutions contain the diethylstil-

bestrol liberated from the conjugate. Treat as for the free stilbestrol from "Wash the ether solution with one-tenth volume of fresh 10 per cent sodium carbonate solution."

Content over 1 mg. per liter. Proceed as for lower contents but stop at the first time the ethanol solution is prepared and is ". . . no more than faintly yellow."

Blood. Dilute with 20 volumes of water, acidify with 1:1 phosphoric acid and proceed as for urine, starting at ". . . cool, and extract thrice with twice the volume of peroxide-free ether."

Procedure—*By phosphotungstic-phosphomolybdic acid.* Add a 10-ml. aliquot of the sample in 30 per cent ethanol to 50 ml. of water and 5 ml. of reagent (Vol. III, page 116). Mix and add 15 ml. of 20 per cent sodium carbonate solution. Dilute to 100 ml. with distilled water, mix, and allow to stand at room temperature for 1 hour. Read at 460 mμ in comparison with results developed from a solution containing 0.02 mg. of diethylstilbestrol per ml. in 30 per cent ethanol.

By nitric acid. Transfer an aliquot containing 0.5-2 mg. of diethylstilbestrol and a corresponding standard to tubes. Evaporate the solvent *in vacuo* in boiling water. Take up in 6 ml. of glacial acetic acid. Add 0.1 ml. of concentrated sulfuric acid and 0.04 ml. of concentrated nitric acid to each of the residues and heat in boiling water for 90 seconds to develop the color. Cool, add 10 ml. of water, and make alkaline with 15 ml. of concentrated ammonium hydroxide. Cool, dilute to 50 ml., and read.

As nitroso compound. Evaporate a portion of sample containing 0.1-0.15 gram of diethylstilbestrol to dryness and take up in 5 ml. of a 1.5 per cent solution of potassium hydroxide in 80 per cent acetic acid. Add 5 ml. of concentrated sulfuric acid and 2 drops of a saturated aqueous solution of sodium nitrite. Dilute to 50 ml. with a 3:2 mixture of ethanol and concentrated ammonium hydroxide. Read at 440 mμ against a reagent blank.

By antimony pentachloride. Evaporate a sample containing 0.05-0.1 mg. of test substance at 80°. Take up in hot chloroform free from alcohol and phosgene and which has been refluxed over Drierite. Cool and dilute to 10 ml. Mix and develop 5 ml. with 6 drops of fresh 50 per cent antimony pentachloride in chloroform. Read at 520 mμ after 3 minutes.

Dihydroxydiethylstilbestrol

Oxidation of dihydroxydiethylstilbestrol gives appropriate colors. This may be by potassium pyroantimoniate or ammonium molybdate in sulfuric acid.[96]

Procedure—To a sample of 1 ml. containing 0.05-0.2 mg. of dihydroxydiethylstilbestrol or an ester in chloroform add 1 ml. of 10 per cent potassium pyroantimoniate in 1:1 sulfuric acid. Shake until the color in the chloroform layer disappears and add concentrated sulfuric acid to 25 ml. Read at 500 mμ against water. The color is stable for several hours.

Hexestrol, Cycloestrol, *p-p'*-(1,2-Diethylethylene)diphenol

The color with α-nitroso-β-naphthol and nitric acid is suitable for estimation of hexestrol.[97] The reaction is also developed with phosphotungstic-phosphomolybdic acid as described for diethylstilbestrol[98] and with diazotized sulfanilic acid.[99]

Samples—*Tablets.* Extract as described for stilbestrol (page 418).
Oil. Extract as described for stilbestrol (page 418).
Urine. Extract as described for stilbestrol (page 418).

Procedure—*By α-nitroso-β-naphthol.* Evaporate an aliquot of sample containing 0.005-0.35 mg. of hexestrol. Take up the residue in 5 ml. of water by shaking and heating. Add 2 drops of 0.1 per cent solution of α-nitroso-β-naphthol in ethanol and shake for 20 seconds. Add 6 drops of concentrated nitric acid from a microburet and shake for 20 seconds. Heat with agitation in boiling water for a minute, cool, and read against a blank in which the nitric acid is replaced by water.

By nitric acid. See page 423.

By diazotized sulfanilic acid. As reagent add 4.5 grams of sulfanilic acid to 4.5 ml. of concentrated hydrochloric acid and 100 ml. of water.

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96 B. Salvadori, *Boll. soc. ital. biol. sper.* **24**, 978-9 (1948); *Ibid.* **26**, 1058-9 (1950).

97 F. H. Malpress, *Biochem. J.* **39**, 95-8 (1945); J. Cheymol and A. Carayon-Gentil, *Bull. soc. chim. biol.* **27** 382-7 (1945); A. Carayon-Gentil and J. Cheymol, *Ann. pharm. franc.* **6**, 129-36 (1948).

98 R. A. Dunford, *Can. J. Research* **27B**, 646-52 (1949).

99 Gino Carrara, *Z. physiol. Chem.* **279**, 117-19 (1943).

Filter and dilute to 500 ml. To 1.5 ml. of this solution, chilled, add 1 ml. of cold 5 per cent sodium nitrite solution. Chill in ice for 10 minutes and dilute to 50 ml.

Develop as described for stilbestrol (page 420).

4,4′-(Diethylidene ethylene)diphenol, Dienestrol

Diazotized sulfanilic acid is a suitable reagent for estimation of dienestrol.[100] The red color obtained is stable for several hours and conforms to Beer's law. It is also developed with vanillin. For development with nitric acid [101] interference by diethylstilbestrol is avoidable.[102]

Samples—*Tablets.* Prepare as for stilbestrol (page 418).

Oils. Free dienestrol. Shake 1-2 ml. of sample containing 2-5 mg. of dienestrol mechanically for 30 minutes with 20 ml. of methanol. Decant the methanol through a filter and reextract the oil. Dilute the filtrate to 50 ml. with methanol.

Urine. Prepare as described for stilbestrol (page 418).

Procedure—*By diazotized sulfanilic acid.* As reagent dilute 9 ml. of 10 per cent sulfanilic acid in concentrated hydrochloric acid to 100 ml. with water. Chill 1.5 ml. of this solution in ice and add 1.5 ml. of 5 per cent sodium nitrite solution. After 5 minutes add 6 ml. more of 5 per cent sodium nitrite solution. Dilute after 10 minutes to 50 ml. and let stand for an hour before using. As buffer for pH 12 dissolve 20 grams of boric acid in 280 ml. of 4 per cent sodium hydroxide solution and dilute to a liter with water.

Shake 1 ml. of sample containing 0.025-0.125 mg. of dienestrol in methanol with 8 ml. of the buffer and heat at 80° for 6 minutes. Cool to 20° and add 2 ml. of reagent. Shake for 30 seconds, let stand for 5 minutes, and read at 500 mμ against a blank in which 2 ml. of water replace the reagent. If turbid add 2 drops of 10 per cent calcium chloride to the sample and filter before reading.

By vanillin. Evaporate a sample containing 0.01-0.1 mg. of dienestrol to dryness. Add 2 ml. of 0.5 per cent vanillin in 1:1 sulfuric acid. Heat in boiling water for 2 minutes, cool, dilute to 5 ml. with water, and read. The blue changes to red on long standing.

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[100] Gino Carrara, *Z. physiol. Chem.* **279**, 117-19 (1943); A. Carayon-Gentil and J. Cheymol, *Bull. soc. chim. biol.* **28**, 723-9 (1946); *Ibid.* **29**, 543-7, 1075-8 (1947); *Ann. pharm. franc.* **6**, 129-36 (1948); F. H. Malpress, *Biochem. J.* **43**, 132-6 (1948).

[101] R. A. Dunford, *Can. J. Research,* **27B**, 646-52 (1949).

By nitric acid. Evaporate to dryness a sample containing 0.5-2 mg. of dienestrol. Cool and take up in 6 ml. of glacial acetic acid. Add 0.1 ml. of concentrated sulfuric acid and 0.04 ml. of concentrated nitric acid. Mix and let stand at room temperature for 1 hour. Add 10 ml. of water, then 15 ml. of concentrated nitric acid, and dilute to 50 ml. for comparison.

Esestrol

Esestrol is determinable by the nitration method for diethylstilbestrol (page 423).

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[102] F. H. Malpress, *Biochem. J.* **39**, 95-8 (1945).

CHAPTER 11

ALKALOIDS[1]

This chapter contains a residue of alkaloids not elsewhere classified. Thus some synthetic alkaloids are determined by methods which automatically classify them as amines. Others are in this chapter. Some alkaloids are also glucosides and have been so grouped, for example, digitoxin and strophanthin. The remainder which appear here are listed alphabetically by their most common names, regardless of a chemical name which in some cases precedes the common name in the title. Sometimes the selection of a common name has to be arbitrary, for example, choosing between marihuana and cannabis.

There are many class reactions applicable to natural alkaloids. It follows that they must ordinarily be isolated before development of color.

Alkaloids in General

The addition of a dyestuff which combines with an alkaloid permits quantitative extraction into a solvent.[2] The dyestuff is usually a sulfonic acid and must be relatively insoluble in the organic solvent. The reaction is, with modifications, not only applicable to alkaloids but also to many other basic synthetic organic compounds, including antihistamines. Cocaine gives low results due to decomposition in distillation during preparation of the sample, as do also pontocaine, a *p*-aminobenzoic acid derivative, and morphine.

Aside from the color with picric acid, one of the most basic methods involves precipitation with silicotungstic acid and measurement of the blue color obtained by treating with titanium chloride.[3] Another is based upon precipitation with Reinecke's salt.[4]

Sample—*Tissue.* Mix 500 grams of macerated tissue, 500 ml. of water, and 2 ml. of saturated aqueous tartaric acid. Steam-distil and collect

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[1] See Chapter 1, Volume III, for details of organization, condensation, etc.

[2] Bernard B. Brodie and Sidney Udenfriend, *J. Biol. Chem.* **158**, 705-14 (1945); Alexander O. Gettler and Irving Sunshine, *Anal. Chem.* 23, 779-81 (1951).

[3] M. Mascré and J. Losieau, *Bull. sci. pharmacol.* **48**, 273-80 (1941).

[4] Duquenois, *Ann. fals.* **32**, 95-7 (1939).

150 ml. of distillate. Proteins are coagulated at the temperature for steam distillation and later extraction of alkaloids is facilitated. Discard the distillate and filter the contents of the flask while hot. Apply a correction for the solids—usually 150 ml. for a 500-gram sample—and use an aliquot of the extract.

Procedure—*By methyl orange.* Add 16 per cent sodium hydroxide solution to the extract until the pH is 7.5-8.2 as shown by an external indicator. Usually 2-4 drops are sufficient. At this pH alkaloids are completely extracted by chloroform, but extraction of organic bases is at a minimum. Shake mechanically with 25 ml. of chloroform for 20 minutes and then centrifuge.

Mix the clarified chloroform extract with 7 ml. of a freshly prepared 1:1 mixture of saturated aqueous methyl orange and saturated boric acid solution. Shake mechanically for 10 minutes and completely separate from the aqueous layer. Mix 10 ml. of the chloroform layer with 1 ml. of 1:50 sulfuric acid-absolute ethanol and read at 520 mμ against a reagent blank. If not sensitive enough, extract the 10 ml. of chloroform extract with 0.5 ml. of 1:25 hydrochloric acid and read this.

By silicotungstic acid. To 1 ml. of solution of the alkaloid, add 7 ml. of 1:10 hydrochloric acid and 2 ml. of 5 per cent silicotungstic acid. Centrifuge and wash the precipitate with a minimum volume of 1:18 hydrochloric acid. Combine the original centrifugate and washings and dilute to 20 ml. As reducing agent mix 1 volume of 15 per cent titanous chloride solution with 9 volumes of 1:5 hydrochloric acid. Add 0.5 ml. of this to a 5-ml. portion of the diluted residual solution and read against a reagent blank. This measures the silicotungstic acid precipitated which varies with different alkaloids. Therefore, only approximate values are obtainable for mixtures.

By ammonium reineckate. As reagent stir mechanically 2 grams of ammonium reineckate in 100 ml. of water for 10 minutes. Filter through hard paper and prepare fresh daily.

Mix a 10-ml. aliquot of the prepared sample solution with 3 ml. of ammonium reineckate reagent and allow to stand in an ice bath for 30 minutes. Filter through a 15-ml. low-form sintered glass crucible and wash with two 5-ml. portions of 1:500 reagent.

Dissolve the precipitate by passing 2-ml. portions of acetone through the crucible and dilute to 10 ml. Read at 525 mμ against acetone and interpret from the curve for the alkaloid known to be present.

Apomorphine

In the absence of interfering phenols, apomorphine is determined with dibromoquinone-chlorimide.[5]

Procedure—To 1 ml. of a solution containing 0.02-0.2 mg. of apomorphine, add 0.1 ml. of a 0.46 per cent solution of 2,6-dibromoquinone-chlorimide in absolute ethanol. Add 0.5 ml. of 5 per cent sodium bicarbonate solution and after 30 minutes extract the color with 2 ml. of butanol. Read the color of the extract against a reagent blank.

dl-Tropyl tropate, *dl*-Hyoscyamine, Atropine

Atropine is the optically inactive form of hyoscyamine. Methods of determination are the same. They form salt-like addition compounds with acidic dyes. These are characterized by their solubility in organic solvents such as benzene. After decomposition with alkali, the dye is estimated colorimetrically.[6] Other alkaloids showing the reaction, and which must therefore be absent for estimation of hyoscyamine, are scopalamine and homatropine. The same method is applied to them. The color is stable for at least 2 hours. Accuracy is better than ±5 per cent. Chloroform may replace benzene for extraction, increasing the sensitivity but also the blank.

Small quantities of atropine couple with *p*-dimethylaminobenzaldehyde in strong sulfuric acid to give a violet color.[7] Atropine after nitration is read in alkaline ketone solution[8] or estimated by precipitation with silicotungstic acid and reduction of the residual silicotungstic acid with titanous chloride.[9]

Another method is by precipitation of the reineckate and either filtering and reading the residual solution or dissolving the precipitate

[5] E. R. Cole, *J. Proc. Roy. Soc. N. S. Wales* **81**, 80-3 (1947).

[6] Frances Durick, J. Stanton King, Jr., Palmer A. Ware, and Georg Cronheim, *J. Am. Pharm. Assoc.* **39**, 680-2 (1950).

[7] R. P. Daroga, *J. Indian Chem. Soc.* **18**, 579-84 (1941); F. H. L. van Os, *Rec. trav. chim.* **64**, 35-40 (1945); K. R. Gottlieb, *Dansk. Tids. Farm.* **24**, 40-8 (1950); K. Jentzsch, *Scientia Pharm.* **20**, 6-16 (1952).

[8] R. San Martin Casamada, Arturo Mosqueira Toribio, and Luis García Boente, *Farmacognosia* **10**, 185-96 (1950).

[9] M. Mascré and J. Loiseau, *Bull. sci. pharmacol.* **48**, 273-80 (1941); A. Romeike, *Pharm. Zentralhalle* **91**, 80-5 (1952).

in acetone[10] or 1:1 acetone-ethanol[11] for reading. The reineckate method is applicable to 2-10 mg. of atropine sulfate, quinine sulfate, strychnine sulfate, emetine hydrochloride, hyoscine hydrobromide, cinchonidine sulfate, cinchonine, quinidine sulfate, sparteine sulfate, hydrastine hydrochloride, cocaine hydrochloride, and pilocarpine nitrate. Ephedrine, colchicine, and morphine are not quantitatively precipitated. Starch, talc, and insoluble stearates do not interfere with the precipitation, development of color, or the clarity of the acetone solution of the reineckate. The picrate in chloroform solution is read in the absence of interfering substances such as urotropine and many other alkaloids.[12]

Samples—*Drug solutions.* Centrifuge to remove chromogens. Evaporate an appropriate sample to a sirup on the water bath and transfer to a filter with 1:360 sulfuric acid. Provided that interfering substances precipitated in evaporation do not redissolve in the acid, adjust the pH of the filtrate to 5.2-5.5 and dilute to a known volume for the use of aliquots. If necessary, remove chlorophyll by the USP technic. Develop with bromocresol purple. The method is not applicable to tincture of hyoscyamus.

Tablets. Reduce 20 or more tablets to a fine powder in a mortar and digest at 100° for 30 minutes with a suitable amount of 1:100 sulfuric acid so that 10 ml. of solution contains about 5 mg. of alkaloid. Filter, wash the residue, and dilute to a known volume for development as the reineckate.

For development with *p*-dimethylaminobenzaldehyde weigh a pulverized tablet and place on filter paper. Extract on a funnel with a minimum volume of 1 per cent aqueous tartaric acid. Extract with 5, 5, and 5 ml. of ether and discard the extracts. Make alkaline with a few drops of 10 per cent sodium hydroxide solution and extract with five successive 5-ml. portions of chloroform. Discard the aqueous layer and filter the chloroform through anhydrous sodium sulfate on cotton. Evaporate to dryness in boiling water and take up in chloroform to give 0.06-0.1 mg. of alkaloids per ml.

Procedure—*By bromocresol purple.* As a buffer dissolve 19 grams of monosodium phosphate monohydrate and 1 gram of anhydrous di-

[10] F. J. Bandelin, *J. Am. Pharm. Assn., Sci. Ed.* 37, 10-12 (1948); *Ibid.* 39, 493-5 (1950).

[11] Maria Rozynska and Krystena Gorzczynska, *Farm. Polska*, 8, 421-2 (1952).

[12] S. M. Bolotnikov, *Aptechnoe Delo* 1, 42-6 (1952).

sodium phosphate in water and dilute to 500 ml. As reagent dissolve 0.2 gram of bromocresol purple in 15 ml. of water and 3.2 ml. of 0.4 per cent sodium hydroxide solution and dilute to 250 ml.

Mix 10 ml. of sample containing 0.1-0.2 mg. of alkaloid with 5 ml. of buffer and 5 ml. of reagent. Extract for 3 minutes with 50 ml. of benzene. Separate the extract and repeat the extraction. Centrifuge the combined extracts to remove aqueous menstruum and extract an 80-ml. aliquot with two 10-ml. portions of 0.2 per cent sodium hydroxide solution. Dilute the combined extracts to 25 ml. with 0.2 per cent sodium hydroxide solution and filter through cotton to clarify. Read at 580 mμ with PC-4 as a secondary filter against a reagent blank.

By p-dimethylaminobenzaldehyde. Evaporate a sample containing 0.2 mg. to dryness. Dry at 120° and cool. Add 7 drops of 10 per cent *p*-dimethylaminobenzaldehyde in 88 per cent sulfuric acid. After 2 minutes place in boiling water for 3 minutes and chill in ice water. Add 5 ml. of acetic anhydride and let stand for an hour. Read at 515 mμ against a reagent blank.

By nitration. Evaporate the atropine solution to dryness and moisten the dry residue with fuming nitric acid. Dry and extract the residue with a few ml. of acetone. Dilute to about 9 ml., add 0.5 ml. of 10 per cent potassium hydroxide in absolute methanol, and dilute to 10 ml. with acetone. Read at once with a green filter against a reagent blank.

Brucine

Brucine gives a reddish yellow color with nitric acid. Strychnine also gives a color on standing. By adding potassium chlorate as a reagent, the interference from strychnine is removed and the color increases gradually on standing, instead of fading.[13] The error of the method is ±1-4 per cent for 5 to 50 mg. of brucine in a mixture of brucine and strychnine.

Sample—*Alkaloid bases.* Dissolve 0.1 gram of sample alkaloid mixture in 20 ml. of 1:200 sulfuric acid with heating. Dilute to 30 ml. with the same solvent.

Alkaloid salts. Render a solution of the salts alkaline and extract

[13] E. Dowzard, *Proc. Chem. Soc. London* **18**, 220-1 (1902); A. Wöber, *Z. angew. Chem.* **31**, 124 (1918).

with chloroform. Evaporate the chloroform extract to dryness. Treat 0.1 gram of the dry residue as given under alkaloid bases.

Procedure—To the sample add 10 ml. of a 1:1 mixture of concentrated nitric acid with 1:7 sulfuric acid. Stir, let stand for one minute, and add 2 ml. of a saturated aqueous solution of potassium chlorate. Stir and dilute to 50 or 100 ml., according to the color intensity. Read at once against a reagent blank.

Mixed Belladonna Alkaloids

The Solanaceae alkaloids, belladonna and stramonium, are determined by precipitation with ammonium reineckate $NH_4[Cr(SCN)_4(NH_3)_2]$ and solution of the washed precipitate in acetone.[14] Results agree with those of the USP method to ± 5per cent. Alternatively develop by treatment with fuming nitric acid and later with potassium hydroxide solution. Results are about 10 per cent lower than those by the USP official method.

Atropine and all the other belladonna alkaloids give a green complex in an organic solvent with naphthenic acid and cupric sulfate.[15] Results are in agreement with those by titration and are accurate to ±0.5 per cent for atropine.

Sample—*Crude drug.* Wet 1 gram of powdered drug with 1 ml. of ethanol and 0.1 ml. of 10 per cent ammonium hydroxide solution. Add 5 ml. of chloroform and boil for 2-3 minutes. Transfer to a miniature percolator plugged with a small wad of chloroform-soaked cotton and suspended in a 60-ml. separatory funnel. Percolate slowly with warm chloroform until at least 30 ml. is collected in the funnel. Extract the chloroform solution with 5 successive 10-ml. portions of 1:72 sulfuric acid. Make the extract alkaline with 1:9 ammonium hydroxide solution and extract completely with chloroform. Remove aliquot portions of the chloroform extract and evaporate nearly to dryness. Add 5 ml. of 1:72 sulfuric acid and evaporate off the balance of the chloroform for development as the reineckate.

For development by nitric acid, after boiling with 5 ml. of chloroform as above, proceed as follows: Transfer to a miniature percolator

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[14] A. B. Colby and J. L. Beal, *J. Am. Pharm. Assoc., Sci. Ed.* **41**, 351-4 (1952).

[15] Lee Worrell and Roger E. Booth, *J. Am. Pharm. Assoc.* **42**, 361-4 (1953).

plugged with a small wad of chloroform-soaked cotton, and suspended in a stoppered 100-ml. graduated cylinder. Percolate slowly with warm chloroform until 30 ml. of percolate are obtained. To this add 6 per cent acetic acid in 5 per cent ethanol, to a total volume of 80 ml. Stopper and shake by inverting 50 times. After separation of the layers, remove 5-10 ml. of the acidic upper layer and filter. Evaporate 1 ml. of filtrate to dryness.

For development with naphthenic acid and cupric salts, add to 10 grams of drug in an extraction thimble a mixture containing 8 parts of concentrated ammonium hydroxide, 10 parts of ethanol, and 20 parts of ether. Stir, cover the drug with the liquid, and let stand overnight in a Soxhlet apparatus. Add ether and extract for 3 hours. Transfer the ether extract to a separatory funnel, using 2 portions of ether and 2 portions of 1:72 sulfuric acid for rinsing. Extract with 15-ml. portions of the sulfuric acid until a negative test is obtained with mercuric iodide solution. Combine the acid portion, make alkaline with ammonium hydroxide, and extract with 15-ml. portions of chloroform until a negative test is obtained. Evaporate the combined chloroform extracts to dryness and heat for 15 minutes longer. Dissolve the residue in chloroform, evaporate again to dryness, and heat for 15 minutes longer. Repeat the chloroform treatment once more. Dissolve the residue in a minimum amount of chloroform, using small portions of benzene and a final rinse with 3 ml. of acetone for transfer. Dilute to 50 ml. with benzene for development of total belladonna drugs with napthenic acid and cupric salts.

Tablets. For total belladonna drugs prepare mercuric iodide test solution—Valser's reagent—by slowly adding a 10 per cent solution of potassium iodide to red mercuric iodide until almost all of the red mercuric iodide is dissolved. Filter to remove excess mercuric iodide.[16]

Dissolve an atropine sulfate tablet in 5 ml. of water and 1 ml. of ammonium hydroxide. Extract with 3 or 4 portions of benzene until a negative test is obtained with mercuric iodide solution. Evaporate the combined benzene extracts to less than 25 ml. Test the vapors with moistened litmus paper and heat until no more ammonia is present. Transfer to a 25-ml. volumetric flask by washing with benzene and dilute to volume with this solvent for development with naphthenic acid and cupric salts.

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[16] *U. S. Pharmacopeia,* 14th Ed., p. 941 (1950).

Procedures—*As the reineckate.* To the acid solution of sample add 10 ml. of a 1 per cent aqueous solution of ammonium reineckate to precipitate the alkaloids as insoluble reineckates. Let stand a half hour, stirring frequently. Filter by suction through sintered glass. Wash the beaker and precipitate with three 2-ml. portions of cold water. Dissolve the precipitate in acetone by passing small portions through the filter and collecting the colored filtrate. Wash the beaker with a little acetone and add to the filtrate. Dilute to 25 ml. with acetone and read at 525 mμ against acetone as a blank. Use hyoscyamine sulfate as standard.

By nitric acid. To the dried residue of sample, add 0.2 ml. of fuming nitric acid, moistening the entire residue, and again evaporate to dryness. Dissolve the residue in acetone and dilute to 25 ml. with acetone. Add 0.1 ml. of 3 per cent potassium hydroxide solution in methanol. Exactly 7 minutes after the color appears, read against an acetone blank, using hyoscyamine sulfate as standard.

By naphthenic acid and cupric salts. To 25 ml. of sample in benzene add 2 ml. of 20 per cent naphthenic acid in benzene and 2 ml. of a 5 per cent aqueous cupric sulfate solution. Mix and dilute to 50 ml. with benzene. Let stand at room temperature for 18 hours or longer. Read the benzene layer at 700 mμ against a blank. In the case of the crude drug sample, the blank is prepared by using the sample solution with all the other additions except cupric sulfate.

1,3,7-Trimethylxanthine, Theine, Methyltheobromine, Caffeine

When caffeine is oxidized and then treated with ammonia it gives a crimson red.[17] Other xanthides such as theobromine and theophylline give the color. Guanine gives a similar reaction. In some cases the colors of other alloxantines are less stable than those of caffeine. Oxidizing agents which are applicable are acidified saturated bromine water or chlorine water, aqueous acidified chloramine T, hydrogen peroxide and acid, and 9:1 concentrated nitric-hydrochloric acid. The reaction has been modified with zinc or mercuric acetate or triethanolamine replacing the ammonium hydroxide.

A very dilute caffeine solution treated with concentrated sodium phosphotungstate solution gives a stable turbidity. Within the range of

[17] Georges Denigès, *Bull. soc. pharm. Bordeaux* **72**, 345-55 (1934); *Ibid.* **73**, 5-7 (1935); Charles E. Morgan and Nicholas Opolonick, *Ind. Eng. Chem., Anal. Ed.* **17**, 526-7 (1945).

0.002-0.005 per cent of caffeine this is suitable for nephelometric estimation to ±1 per cent.[18] Temperature must be closely controlled.

Caffeine can be read in the ultraviolet.[19] Maximum absorption takes place at 272 mμ at pH 5-9. All substances in coffee extracts and crude caffeine which absorb light at 272 mμ must be removed, as by zinc ferrocyanide followed by magnesium oxide. Magnesium oxide removes chlorogenic acid and zinc ferrocyanide removes trigonelline. The reagents have a slight but negligible absorption at 272 mμ. The method is satisfactory for biological material because the transformation products of caffeine do not interfere.[20] The clarification step does not remove all interfering substances in roasted coffee. The use of potassium permanganate [21] in neutral solution is a necessary supplement.

Caffeine is read directly at 6.04 μ.[22] Interference by acetylsalicylic acid (Vol. III, page 413), phenacetin, codeine, or threnylpyramine is avoidable. It is also read on drug solutions in the ultraviolet with correction for phenacetin (page 205). The sample so used desirably contains about 32 mg. of caffeine.

Samples—*Solutions.* For oxidation and development evaporate the sample solution containing 0.1-1 mg. of caffeine in ethanol to dryness by gentle heating. Add 6 drops of saturated bromine-water and 1 drop of concentrated hydrochloric acid to the residue. Dilute with water to 2 ml. Rotate the dish with the contents over the point of a flame so that the liquid is evaporated to dryness. Continue to heat until the residue has turned to a reddish orange color. There should be no yellow and no decomposition. This operation is delicate and requires careful manipulation. Develop with mercuric acetate.

Coffee beans or leaves. Grind the sample and weigh out 0.25 gram

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18 Erich Herndlhofer, *Mikrochemie* **12**, 227-30 (1932).

19 A. Castille and E. Ruppol, *Bull. soc. chim. biol.* **10**, 623-66 (1928); Ensor R. Holiday, *Biochem. J.* **24**, 619-25 (1930); J. M. Gulland, E. R. Holiday, and T. F. Macrae, *J. Chem. Soc.* **1934**, 1639-44; John R. Loofbourow, Miriam Michael Stimson, and Sr. Mary Jane Hart, *J. Am. Chem. Soc.* **65**, 148-51 (1943); N. H. Ishler, T. P. Finucane, and Emanuel Borker, *Anal. Chem.* **20**, 1162-6 (1948);

20 Julius Axelrod and Jules Reichenthal, *J. Pharmacol. Exptl. Therap.* **107**, 519-23 (1953).

21 K. Lendrich and F. E. Nottbohm, *Z. Nahr. Genussm.* **17**, 241 (1914); G. Fendler and W. Stüber, *Ibid.* **28**, 9 (1941); Association Official Agr. Chem., *J. Assoc. Official Agr. Chem.* **30**, 70-1 (1947).

22 T. V. Parke, A. M. Ribley, E. E. Kennedy, and W. W. Hilty, *Anal. Chem.* **23**, 953-7 (1951).

of the fine powder. Digest with about 25 ml. of water for 30 minutes. Extract with two 20-ml. portions of chloroform. Filter the combined chloroform extracts and evaporate the solvent. Dissolve the caffeine in 10 ml. of hot water, cool, filter, and dilute to 50 ml. for development by oxidation.

Crude caffeine in the ultraviolet. Add 400 ml. of hot water to 0.1 gram of sample and shake thoroughly. Cool to room temperature and dilute to 500 ml. with water. Dilute an aliquot containing 2 mg. of caffeine to a volume of 50 ml. with water. Add 7 ml. of 22 per cent zinc acetate dihydrate solution in 3 per cent acetic acid and swirl vigorously. Then add 6 ml. of 10.5 per cent potassium ferrocyanide solution dropwise with continuous swirling. Dilute to 100 ml. with water, mix thoroughly, and filter, discarding the first 10-ml. portion. Add a 50-ml. aliquot to 5 grams of heavy magnesium oxide USP. Add 50 ml. of water and boil for 20 minutes. Cool to room temperature and adjust to a weight of 105 grams plus tare. Filter and discard the first 10-ml. portion.

Green coffee in the ultraviolet. To 2 grams of sample add 50 ml. of 1:360 sulfuric acid and 450 ml. of water. Boil for 30 minutes. Cool to room temperature and adjust to a weight of 502 grams plus tare. Filter and use a 50-ml. aliquot for clarification. Proceed as under crude caffeine, starting at "Add 7 ml. of 22 per cent zinc acetate . . .."

Caffeine benzoate.[23] Dilute a sample of appropriate concentration, but not over 5 mg., to 100 ml. with 1 per cent phosphoric acid in 95 per cent ethanol. Read in the ultraviolet.

Antihistamines. See the separation under that topic (page 280).

Procedure—*By mercuric acetate.* Add 10 ml. of distilled water to the residue in the dish. Add 1 drop of a 5 per cent solution of mercuric acetate in 1:50 acetic acid. Stir until the residue dissolves, which takes place readily. Transfer the red solution to a test tube and read against a reagent blank.

By zinc acetate. Follow the procedure for mercuric acetate but use 5 per cent zinc acetate solution in 1:50 acetic acid and also add 1 drop of glacial acetic acid.

By phosphotungstic acid. To 20 ml. of the sample solution add 1 ml.

[23] Hector R. Hernandez and Albert M. Mattocks, *Bull. Natl. Formulary Comm.* **19**, 1-3 (1951).

of a 20 per cent solution of sodium phosphotungstate in 1:17 hydrochloric acid. Mix and read after a few minutes.

In the ultraviolet. Read the solution at 272 mμ for caffeine. If benzoate is present also read at 228 mμ and calculate from $87A_{272} - 7A_{228}$.

Cinchonidine

A general method applicable to cinchonidine is the formation of molecular complexes of organic bases with sulfonic acids, which complexes are soluble in organic solvents for reading.[24] The method is also applicable to the specific cinchona alkaloids in the absence of other members of the family. The complex of cinchonidine with methyl orange is highly soluble in either ethylene dichloride or chloroform. A complex extraction of the alkaloid into the organic solvent is first necessary.

Cinchonidine is determined as the reineckate by a technic described under alkaloids in general (page 428). Alternatively, read cinchonidine along with cinchonine in the ultraviolet. The method is given under quinine (page 477).

Sample—*Plasma.* Mix 1-5 ml. of plasma with 1 ml. of 4 per cent sodium hydroxide solution and 20 ml. of ethylene dichloride. The pH must exceed 9. Shake for 5 minutes mechanically, decant, and centrifuge for 10 minutes to break the emulsion. If a solid emulsion forms, break by stirring and recentrifuge. Aspirate off the aqueous layer. Add an equal volume of 0.56 per cent potassium hydroxide solution to the ethylene dichloride layer and shake mechanically for 10 minutes. Centrifuge and aspirate off the aqueous layer for determination by methyl orange.

Urine. Dilute urine to contain 0.001-0.004 mg. of cinchonidine per ml. Mix 1 ml. with 1 ml. of 4 per cent sodium hydroxide solution and 20 ml. of ethylene dichloride. Shake mechanically for 5 minutes, let the phases separate, and aspirate off the aqueous layer. Add an equal volume of 10 per cent sodium hydroxide solution to the solvent layer and shake mechanically for 5 minutes. Again allow to separate and remove the aqueous layer. Add a few ml. of water and mix to dilute the alkaline solution. Aspirate off the separated aqueous layer. Centrifuge for 2 minutes and again aspirate. Develop by methyl orange.

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[24] Bernard B. Brodie and Sidney Udenfriend, *J. Biol. Chem.*, **158**, 705-14 (1945).

Feces. Add 20 ml. of concentrated hydrochloric acid to a sample of feces and dilute with water. Shake until homogenous and treat an aliquot as a plasma sample but omit the washing with alcoholic potassium hydroxide.

Procedure—To an ethylene dichloride solution of sample add 0.5 ml. of saturated methyl orange in 3.1 per cent boric acid solution. Shake for 5 minutes and centrifuge for 5 minutes. Aspirate off the aqueous layer and recentrifuge the solvent layer for 5 minutes. Mix 10 ml. of the solvent layer with 1 ml. of a mixture of 1 ml. of concentrated sulfuric acid and 50 ml. of absolute ethanol. Mix and read at 540 mμ against a reagent blank.

Cinchonine

In the absence of other alkaloids precipitated by picric acid, cinchonine is determined by precipitation, dissolving the cinchonine picrate in ammonium hydroxide, and estimating the picric acid in the soluton.[25] The method is accurate to ±2.5 per cent. Cinchonine is precipitated quantitatively with silicotungstic acid. By dissolving this precipitate, the tungsten can be reduced to tungsten blue and used for colorimetric estimation. The method is accurate within ±1 per cent. Cinchonine is precipitated with phosphotungstic acid and estimated as tungsten blue. The method is similar to that with silicotungstic acid. Other substances such as strychnine, quinine, and emetine interfere. The method is accurate to ±2 per cent. Cinchonine and cinchonidine are read in the ultraviolet by a method given under quinine. Bromothymol blue reacts with cinchona alkaloids to give colors which are extractable with benzene for determination[26] (page 477). Cinchonine is determined by precipitation as the reineckate by a method described under alkaloids in general (page 428). The method by silicotungstic acid is also applicable.

Sample—*Blood.* Lake 1 ml. of oxalated blood with 29 ml. of water. Add 5 ml. of 4 per cent sodium hydroxide solution and heat for 30 minutes on a steam bath. Shake thoroughly with 2 ml. of acetone and 50 ml. of ether and discard the aqueous layer. Wash the ether extract with 50 ml. of 0.28 per cent potassium hydroxide solution and then with 50 ml. of water. Add 2.5 ml. of 1:240 hydrochloric acid to the ether

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[25] C. A. Rojahn and Rudolf Seifert, *Arch Pharm.* **268**, 499-520 (1930).

[26] P. B. Marshall and E. W. Rogers, *Biochem. J.* **39**, 258-60 (1945).

and shake. Repeat the acid extraction of the ether and heat the combined extracts on a steam bath to evaporate the dissolved ether. Develop all or an aliquot with bromothymol blue.

Procedure—*By picric acid.* Mix a 5-ml. sample containing 1 to 5 mg. of cinchonine per ml. with 5 ml. of 1 per cent picric acid solution. After 24 hours, centrifuge and decant. Disperse the precipitate in 5 ml. of water. Centrifuge and decant. Repeat. Add 5 ml. of 1:6.5 ammonium hydroxide to each and warm to decompose the cinchonine picrate. Centrifuge and decant the picrate solution. Wash the residues well and add the washings. Dilute to 100 ml. Add 5 ml. of chloroform and shake well. When the chloroform separates, determine the cinchonine by reading the color of the supernatant layer.

By silicotungstic acid. Mix 5 ml. of sample solution containing 1 to 5 mg. of cinchonine per ml., 5 ml. of 1:18 sulfuric acid, 1 ml. of 10 per cent sodium chloride solution, and 2.5 ml. of 10 per cent silicotungstic acid. Mix well and let stand for 3 hours with occasional stirring. Centrifuge and decant. Disperse the precipitate in 2.5 ml. of 1:18 sulfuric acid and 0.5 ml. of 10 per cent sodium chloride solution. Centrifuge and decant. Repeat the washing operation. This removes excess silicotungstic acid. Add 5 ml. of 1:6.5 ammonium hydroxide to the precipitate and heat on a water bath to decompose. Cool, filter, and dilute to 50 ml. Dilute 5 ml. of the prepared solution of the sample to 20 ml. and add 1 ml. of 10 per cent gum arabic solution and 1 ml. of 1:10 hydrochloric acid. Add 2 ml. of titanous chloride reagent (page 428) and dilute to 25 ml. Read against a reagent blank.

By phosphotungstic acid. Use the method for quinine (page 476).

By bromothymol blue. Add 1 ml. of 0.04 per cent bromothymol blue solution to the aqueous sample. Adjust to pH 7 with 1 per cent sodium hydroxide solution. Add 1 ml. of buffer for pH 7 (Vol. I, page 174). After 1 hour add 1 ml. of benzene. Shake mechanically for 40 minutes and centrifuge. Remove the benzene layer and read at 600 mμ against a reagent blank.

Benzoylmethylecgonine, Cocaine

The occasion to determine cocaine is often in the presence of procaine. Since they have similar solubilities, a suitable technic is in the form of decomposition products. Cocaine is hydrolyzed to methanol

and that oxidized to formaldehyde.[27] Lactose, starch, and cane sugar do not interfere. The method will determine 2 mg. of cocaine hydrochloride.

Cocaine in admixture with procaine is also determined by its color reaction with β-naphthol.[28] Cocaine in organic solvent forms quininoids with several dyes which are appropriate for reading.[29] Chloroform is the preferable solvent and bromocresol purple the dye. The solution must be anhydrous. Readings are at 410 mμ with that dye. Cocaine is also determined as the reineckate by the technic described under alkaloids in general (page 428).

Procedure—Add 2 ml. of 2 per cent sodium hydroxide solution and 5 ml. of water to 25-50 mg. of alcohol-free sample. Distil off 2 ml. or more and add 0.25 ml. of 24 per cent ethanol and 2.75 ml. of water. Add 2 ml. of a solution composed of 3 grams of potassium permanganate, 15 ml. of sirupy 85 per cent phosphoric acid, and 100 ml. of water. Allow to stand for 10 minutes and add 2 ml. of a 5 per cent solution of oxalic acid in 1:1 sulfuric acid. Add 5 ml. of Schiff's reagent (Vol. III, page 251), stopper tightly at once, and invert 3 times. After 1-2 hours read at 560 mμ against a reagent blank.

Codeine, Methylmorphine

Codeine as the phosphate is read directly at 10.62 μ.[30] There is no interference by acetylsalicylic acid, phenacetin, caffeine, or threnylpyramine.

The determination of codeine by oxidation with bromine[31] is accurate to ± 3 per cent. Codeine is also oxidized with permanganate and coupled with diazotized sulfanilic acid for reading.[32] Thebaine and narcotine interfere.

[27] James L. Young, *J. Assoc. Offic. Agr. Chemists* **31**, 781-3 (1948).

[28] S. N. Chakravarti and M. B. Roy, *Current Sci.* **6**, 219-20 (1937).

[29] L. A. Woods, J. Cochin, E. J. Fornefeld, F. G. McMahon, and M. H. Seevers, *J. Pharmacol. Exptl. Therap.* **101**, 188-99 (1951).

[30] T. V. Parke, A. M. Ribley, E. E. Kennedy, and W. W. Hilty, *Anal. Chem.* **23**, 953-7 (1951).

[31] N. Yarashevskii, *Org. Chem. Ind.* (USSR) **3**, 29-32 (1937); O. Soboleva, *Farmats. i Farmakol.*, 1937, No. 1, 34-9; Z. M. Vaisberg, Ya. A. Failkov, and E. G. Khrizman, *Farmatsiya* **10**, 26-30 (1947).

[32] Erich Wegner, *Pharmazie* **5**, 33-5 (1950).

Procedure—*By bromine. Drugs.* Dissolve 1 gram in 1 ml. of 1:120 hydrochloric acid and 25 ml. of water. Add a 3.5 per cent solution of bromine in water dropwise until a yellow color persists and add 0.5 ml. excess. Decolorize the solution after 2 minutes by addition of 0.6 per cent sodium sulfite solution and add 1 ml. in excess. Heat at 75-80° for 4 minutes. Cool and add 5 drops of concentrated ammonium hydroxide. After 3-4 hours the color changes from violet-red to yellow-red. Dilute to 50 ml. and read against a reagent blank.

By diazotized sulfanilic acid. Add 0.5 ml. of 3 per cent potassium permanganate solution to 3 ml. of a solution containing not more than 0.5 mg. of codeine. After exactly 15 seconds, add the mixture to 0.5 ml. of freshly prepared 10 per cent ferrous ammonium sulfate solution. Let stand for a few minutes, add 2 ml. of 15 per cent sodium hydroxide solution, and let stand for an hour. Filter and couple 3 ml. of the filtrate with 2 ml. of diazotized sulfanilic acid (page 419). Read at once at 500 mμ.

Colchicine

When an excess of hydroxylamine hydrochloride and sodium hydroxide solution is added to an aqueous solution of colchicine containing sodium salicylate, an orange color develops on standing.[33] The color is stable and can be read at 500 mμ.[34] Aldehydes and ketones are likely to interfere and must be removed in the extraction procedure.

Colchicine is converted to colchiceine by acid hydrolysis; the latter reacts with ferric chloride to give a green color measured at 470 mμ.[35] Colchiceine must be absent in the original sample. Interference from salicylate—commonly present in colchicine products—is removed by addition of sodium chloride or sodium iodide. The method is accurate to about ±5 per cent.

Sample—*Solids.* Extract a powdered sample expected to contain 0.02 gram of colchicine with 20 ml. of chloroform. Let settle and decant. Repeat four times, wash well with chloroform, and evaporate the filtrate to dryness. Take up the residue with 3 successive 15-ml. portions of boiling water, cool, and dilute to 50 ml. for development of aliquots by hydroxylamine hydrochloride.

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33 Kippenberger, *Nachw. Giftstoffen* **1897**, 104.
34 Harry Mack and Edward J. Finn, *J. Am. Pharm. Assoc.* **39**, 532-4 (1950).
35 J. Stanton King, *Ibid.* **40**, 424-7 (1951).

Solutions. Dilute to about 0.5 mg. per ml. Make alkaline to litmus with 2 per cent sodium hydroxide solution and extract 100 ml. with three successive 25-ml. portions of chloroform. Evaporate these to dryness. Take up with 10 ml. of boiling water as sample for development by hydroxylamine hydrochloride.

Pharmaceutical products. Test for the presence of colchiceine by adding, to 5 ml. of a 1 per cent solution of colchicine, 2 drops of a ferric chloride solution containing 9 grams per 100 ml.,[36] and heat. If the solution becomes brownish-red changing to dark brown, colchiceine is present and the method is not applicable.

To 20 ml. containing about 0.5-0.8 mg. of colchicine and no colchiceine, add 1:10 hydrochloric acid until any salicylic acid begins to precipitate. This occurs at about pH 4.2. If sodium iodide is not originally present in the sample, add 2 grams. Dilute with water to 40 ml. and extract with three 30-ml. portions of chloroform. Wash the combined chloroform extracts with 15, 10, and 10-ml. portions of water to remove iodide and any sodium cacodylate which may be present. Evaporate to dryness.

Add 18 ml. of 1:10 hydrochloric acid. Warm on a steam bath without loss of solvent for one hour to hydrolyze the colchicine to colchiceine. Let stand in ice water until all excess salicylic acid has precipitated and then filter. Bring the filtrate to 20 ml. by successive washings with 1:10 hydrochloric acid.

Procedure—*By hydroxylamine hydrochloride.* To 10 ml. of sample solution containing 1-4 mg. of colchicine, add 1 mg. of sodium salicylate and dilute to 15 ml. with water. Add 2 ml. of 2 per cent aqueous hydroxylamine hydrochloride solution and 1.5 ml. of 2 per cent sodium hydroxide solution. Mix thoroughly and heat at 75° for 5 minutes. Chill to room temperature and read at 500 mμ against a reagent blank.

As colchiceine by ferric chloride. To 5 ml. of sample solution corresponding to 0.01-0.1 mg. of colchicine in 1:10 hydrochloric acid, add 0.1 ml. of a 5 per cent solution of ferric chloride and mix. Read against a blank at 470 mμ.

Dihydrocodeinone, Dicodid

Methylene groups which react with *m*-dinitrobenzene in alkaline solution to produce a strong red color must be activated by the presence

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[36] *United States Pharmacopeia*, 14th Ed., pp. 154, 938 (1950).

of an electron-attracting group.[37] Ketones of morphine and codeine contain a methylene group activated by an adjacent carbonyl group.

They are also estimated by the yellow color developed with dimethylaminobenzaldehyde.[38] In an intermediate stage the residue on evaporation is red.

Procedure—*By m-dinitrobenzene.* Dissolve 0.3-1.5 mg. of the sample in 3 ml. of ethanol. Add 4 ml. of 1 per cent *m*-dinitrobenzene solution in ethanol and follow with 4 ml. of 20 per cent sodium hydroxide solution. Dilute to 10 ml. with ethanol and, after 5-10 minutes, read at 500 mμ against a reagent blank.

By dimethylaminobenzaldehyde. As reagent dissolve 0.5 gram of dimethylaminobenzaldehyde in ethanol, add 1 ml. of concentrated sulfuric acid drop by drop, and dilute to 100 ml. with ethanol. Evaporate a known amount of sample to dryness on a water bath. To this add 0.5 ml. of the reagent and dissolve in 10 ml. of 70 per cent ethanol. Read against a sample blank and compare with a dilaudid curve.

Dihydromorphinone, Dilaudid

The reaction is the same as described for dihydrocodeinone.

Emetine

In the absence of other alkaloids precipitated by picric acid emetine is estimated directly from its picrate precipitate or indirectly by the picric acid not precipitated.[39] The methods are accurate to ±2.5 per cent. It also forms a precipitate with silicotungstic acid which can be decomposed to tungsten blue. The method is accurate to ±2 per cent. Emetine is precipitated by phosphotungstic acid and estimated by the tungsten blue in the same way as in the method with silicotungstic acid. Other substances precipitated by phosphotungstic acid such as strychnine, quinine, and cinchonine interfere. The method is accurate to ±2 per cent. Another reaction applied is coupling with diazotized sulfanilic acid to form an azo dye[40] (page 424). Yet another is as the reineckate, the technic being described under alkaloids in general (page 428).

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[37] Teodor Canbäck, *Farm. Revy* **46**, 802-4 (1947); *Collectanea Pharm. Suecica* **11**, 3 pp. (1947) (Pub. 1948).

[38] Juan A. Sanchez, *Semana méd.* (*Buenos Aires*) **1936**, II, 425-6, 713-4.

[39] C. A. Rojahn and Rudolph Seifert, *Arch. Pharm.* **268**, 499-520 (1930).

[40] Yu. N. Rozenblyum, *Farmatsiya* **8**, No. 2, 21-5 (1945).

Procedure—*By picric acid. Direct.* Mix a 5-ml. sample containing 1 to 5 mg. of emetine per ml. with 1 ml. of 1:18 sulfuric acid and 5 ml. of 1 per cent picric acid solution. Centrifuge after 24 hours. Decant and disperse the precipitate in 1.5 ml. of water. Centrifuge and repeat the washing. Decant and dissolve the precipitate in 10 ml. of 1:6.5 ammonium hydroxide. Dilute to 50 ml. and read at 460 mμ against a reagent blank.

Indirect. Mix a 5-ml. sample containing 1 to 5 mg. of emetine per ml. with 2 ml. of 1:18 sulfuric acid and 5 ml. of 1 per cent picric acid solution. Dilute to 50 ml. and let stand for 24 hours at room temperature. After 24 hours filter the solution of sample. To 10 ml. of filtrate add 10 ml. of 1:6.5 ammonium hydroxide and dilute to 100 ml. Read at 460 mμ.

By silicotungstic acid. Mix 5 ml. of sample solution containing 1 to 5 mg. of emetine per ml. with 1 ml. of 10 per cent silicotungstic acid solution. Let stand for 3 hours with occasional stirring. Centrifuge and decant. Prepare a wash liquid from 60 ml. of water, 10 ml. of 10 per cent sodium chloride solution, and 10 ml. of 1:5 hydrochloric acid. Disperse the precipitate in 3 ml. of this, centrifuge, and decant. Repeat this washing procedure to remove excess silicotungstic acid. To the precipitate add 2 ml. of 8 per cent sodium hydroxide solution and warm. When cool add 10 ml. of ether and shake to extract alkaloid. Remove the ether and dilute the aqueous layer to 50 ml.

Mix 5 ml. of the aqueous sample with 1 ml. of 1:10 hydrochloric acid and dilute to 20 ml. Add 1 ml. of titanous chloride reagent (page 428) and dilute to 25 ml. Read against a reagent blank.

By phosphotungstic acid. Follow the procedure given for quinine (page 476).

Ergot Alkaloids

The active alkaloids in ergot include at least ergotoxine, $C_{35}H_{41}N_5O_6$; ergotamine, $C_{33}H_{35}N_5O_5$; ergotaminine, an isomer of ergotamine; and ergotaninine, a dehydration product of ergotoxine by loss of one molecule of water. The first two are the most active. There is no appreciable difference between the reactions of salts of ergotamine and those of ergotoxine. Commercial extracts may contain only ergotinine and ergotoxine. A general reaction with dimethylaminobenzaldehyde determines

the four cited by a violet-blue color.[41] The color is fixed by addition of a definite amount of oxidizing agent followed by a small amount of reducing agent in addition to the reagents.[42] The same technic is also applied to fractions separated as one of the ergot alkaloids.

Many other aldehydes may replace dimethylaminobenzaldehyde, such as vanillin, piperonal, paraldehyde, benzaldehyde, *p*-acetylaminobenzaldehyde, *m*- and *p*-nitrobenzaldehyde, and *m*-aminobenzaldehyde. The colors with these vary from green to purple. Most of these are less sensitive than *p*-dimethylaminobenzaldehyde.

By the presence of a trace of ferric chloride in the sulfuric acid used, the color is completely developed within 1 minute without exposure to light. The heat developed by addition of the reagent furnishes the necessary temperature. Peroxide in the ether used for extraction will cause low results.

Interference by the yellow pigment present in ergot is avoided by suitable manipulation. Substances which occur in ergot extracts which do not interfere are histamine, tyramine, ergosterol, acetyl choline, and choline hydrochloride. Excess of oxidizing agents—such as hydrogen peroxide, sodium nitrite, and ferric chloride—or reducing agents—such as hydrogen sulfide, sodium sulfite, and sodium cyanide—interfere. There is no interference from hydrochloric acid, tartaric acid, sodium hyposulfite, sodium acid phosphate, sodium thiosulfate, tryptophan, indole, or skatole.

Desirably color comparison is with ergonovine maleate as standard. Only acidified alkaloidal extracts should be stored overnight, with refrigeration. Inactive alkaloids, if any occur in ergot, are not degrada-

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[41] H. W. Van Urk, *Pharm. Weekblad* **66**, 473-81 (1929); Maurice I. Smith, *U. S. Pub. Health Repts.* **45**, 1466-81 (1930); Maurice I. Smith and E. F. Stohlman, *J. Pharmacol.* **40**, 77-96 (1930); N. L. Allport and T. T. Cocking, *Quart. J. Pharm. Pharmacol.*, **5**, 341-6 (1932); C. K. Geycart, *J. Assoc. Official Agr. Chem.* **20**, 566-8 (1937); Ralph G. Smith, *Bull. Natl. Formulary Comm.*, **16**, 43-8 (1948); I. Corubolo and M. Grims, *Farm. Glasnik.* **5**, 213-22 (1949); L. Fuchs and W. Himmelbauer, *Scientia Pharm.* **18**, 93-104 (1950); John W. Strong and F. A. Maurina, *J. Am. Pharm. Assoc.* **42**, 414-20 (1953); Kazutaka Yamaguchi, Toyohiko Kawatani, Toshikazu Tabata, Seigo Fukushima, and Miyoko Ito, *J. Pharm. Soc.* Japan **73**, 268-71 (1953).

[42] Károly Száhlender, *Ber. ungar. pharm. Ges.* **13**, 533-5 (1937); A. D. Rosenfeld and M. Ya. Tropp, *Trans. Ukrain, Inst. Exptl. Pharm.* **1**, 79-82 (1938); Elemér Schulek and Gábor Vastagh, *Magyar Gyógyszerés-ztud. Társaság-Értesitöje* **15**, 322-5 (1939); *Dansk. Tids. Farm.* **13**, 101-5 (1939).

tion products of the active alkaloids and do not show up by this method which is accurate to 5 per cent.

Ergot alkaloids give a blue color with sulfuric acid which, extracted from ether solution with sodium carbonate, give a red color for estimation.[43]

Sample—*Powdered ergot. Total alkaloids.* Shake for an hour 20 grams of powdered ergot with 200 ml. of chloroform containing 9 per cent of methanol and 1 per cent of ammonia, and filter. Evaporate 100 ml. of the filtrate at room temperature to 25 ml. and add 75 ml. of ether. Extract with four 15-ml. portions of 1:100 sulfuric acid, using 3-ml. additions of ethanol where necessary to control emulsions. Wash each portion with 50 ml. of ether and dilute the combined extracts to 100 ml. with water. Determine by *p*-dimethylaminobenzaldehyde.

Fluid extract of ergot. Evaporate the alcohol from a 5-ml. sample on a water bath under a current of air. Avoid excess heating. Transfer the sirupy residue quantitatively with 50 ml. of water to a separatory funnel. Add 2 ml. of 1:10 ammonium hydroxide and test to make sure that the suspension is distinctly alkaline to litmus. If necessary, add 1 ml. more of 1:10 ammonium hydroxide. Extract successively with 40, 25, 20, and 15 ml. of ether, free from peroxides. Combine the ether extracts and wash with three 25-ml. portions of water, each containing 0.5 ml. of 1:10 ammonium hydroxide. This washing removes most of the yellow pigment which was extracted with the ether. Wash with two 25-ml. portions of water to remove ammonium hydroxide. Dilute the ether solution of sample to 100 ml. It may be stored for several weeks if tightly stoppered and protected from light.

Extract 50 ml. of the ether extract of ergot alkaloids successively with 10, 10, and 5 ml. of 1 per cent tartaric acid solution. Evaporate the aqueous solution on a water bath to about 15 ml. to free it from ether and dilute to 20 ml. for development with *p*-dimethylaminobenzaldehyde.

Rye flour. Dry 10 grams of rye flour at 110° for 1 hour. Add a mixture of 500 ml. of chloroform and 60 ml. of ethanol. Mix well and, after 1 hour, filter on a folded filter and transfer the residue to the filter. Dry the residue in an oven for 10-15 minutes, to remove chloroform and alcohol completely. To the residue add 30 ml. of ether and 1

[43] F. S. Okolov, *Z. Untersuch. Lebensm.* **57**, 63-71 (1929); Ole Bartsch, *Dansk Tids. Farm.* **5**, 172-5 (1931).

ml. of 1:3 sulfuric acid. Shake occasionally for a day. The residue is mostly gluten. Transfer the ether solution to a filter. Wash the residue and filter with ether until 40 ml. of filtrate have been collected. It will usually be necessary to refilter the first part of the ether solution. Develop by extraction with sodium carbonate.

Wheat flour. Use 20 grams as sample. Follow the method of preparation for rye flour, using 50 ml. of ether and 5 ml. of 1:3 sulfuric acid. In that case the final dilution of the ether solution should be 60 ml.

Procedure—*By p-dimethylaminobenzaldehyde.* As a reagent dissolve 0.20 gram of *p*-dimethylaminobenzaldehyde and 0.03 ml. of 20 per cent ferric chloride solution in a cold mixture of 35 ml. of water and 65 ml. of concentrated sulfuric acid.

Total alkaloid. To 1 ml. of sample in aqueous tartaric acid, add 2 ml. of reagent and read at 610 mμ or 550 mμ against a reagent blank. Interpret in terms of an ergotamine curve or an ergonovine maleate curve. To convert the results for ergonovine maleate, multiply by 1.61 for ergotoxine ethanesulfonate or by 1.37 for ergotoxine alkaloid itself.

Water-soluble alkaloid. Dissolve 1 gram of powdered ammonium sulfate in 8 ml. of sample solution. Add 1:10 ammonium hydroxide until alkaline to litmus. Dilute to 10.6 ml. with water, store in a refrigerator for 12 hours, and filter. Discard the first 3 ml.

Water in soluble alkaloid. Subtract the water-soluble fraction from the total alkaloid.

By extraction with sodium carbonate. Add 2 ml. of saturated sodium carbonate solution to the ether solution of the sample pretreated with sulfuric acid and shake thoroughly. The ergot is extracted into the aqueous alkali layer and gives a rose color. The ether solution should be nearly colorless. Read against a reagent blank.

Ergometrine, Ergonovine

This ergot alkaloid is determined with *p*-dimethylaminobenzaldehyde after extraction to separate from other members of the group.[44] It is also determined by a difference method from ergotoxine, The method of separation removes ergotoxine-like and ergometrine-like alkaloids. Ergometrine is precipitated by picric acid only at high concentrations

[44] Svend Aage Schou and I. Bennekou, *Ibid.* 12, 257-85 (1938); C. E. Powell, O. W. Reagan, Asa Stevens, and Edward E. Swanson, *J. Am. Pharm. Assoc.* 30, 255-9 (1941).

while other alkaloids precipitate readily.[45] Therefore this offers another method of isolation.

Sample—*Ergot.* Shake 12.5 grams of defatted ergot mechanically with 125 ml. of acetone and 2.5 ml. of 1:2.5 ammonium hydroxide for 1 hour. Filter and evaporate 100 ml. in a current of air to approximately 25 ml. Add 10 ml. of water and 10 per cent tartaric acid solution to pH 3. Shake with 50 ml. and 50 ml. of ether to remove fat and coloring matter. Remove the last traces of ether from the aqueous-acetone by vacuum distillation. Adjust to pH 8 with sodium bicarbonate and let stand for 10 minutes. Filter and wash the filter paper with 100 ml. and 10 ml. of 1 per cent sodium bicarbonate solution. The precipitate contains water-insoluble ergotoxine-like alkaloids for separate determination. The filtrate contains the water-soluble ergonovine.

Shake the filtrate with 50 ml. and 50 ml. of carbon tetrachloride to remove all ergotoxine-like alkaloids and then remove all traces of carbon tetrachloride by partial vacuum distillation. Saturate the aqueous layer with sodium chloride and shake with successive portions of diethyl ether until there is no blue color from the aqueous layer with *p*-dimethylaminobenzaldehyde reagent. Evaporate the ether from the combined extracts in a current of air. Take up the residue in 10 ml. of 1 per cent tartaric acid as sample.

Fluid extract of ergot. Shake 12.5 ml. of fluid extract for 1 hour with 10 ml. of 1:2.5 ammonium hydroxide and 125 ml. of acetone. Filter and evaporate 100 ml. of filtrate to dryness in a current of air. Take up the residue in 25 ml. of water and add sodium bicarbonate to pH 8. Add 7.5 grams of sodium chloride and extract with 50, 50, and 50 ml. of ether. Add 25 ml. of 2 per cent aqueous tartaric acid and evaporate the ether from the combined extracts in a stream of air, the tartaric acid solution taking up the alkaloid. Continue as for ergot, starting at "Shake with 50 ml. and 50 ml. of ether . . . ."

Ergotoxine-ergometrine mixtures. The ergotoxine was separated from such a mixture (page 449), leaving the ergometrine in aqueous solution at pH 4. Add 1:1 ammonium hydroxide until distinctly alkaline and extract with ether in a continuous liquid-liquid extractor for 8 hours. Extract the separated ether solution of ergometrine with 10, 10, and 10 ml. portions of 1 per cent tartaric acid solution. Drive off the

[45] Emilio Travucchi, *Boll. soc. ital. biol. sper.* 12, 232-4 (1937).

ether from the acid extract by heating and dilute to a volume to contain 0.005-0.02 mg. of ergometrine per ml.

Procedure—Develop 1 ml. with *p-dimethylaminobenzaldehyde* as described for ergot alkaloids (page 447).

Ergotamine

When ergotamine is separated from other ergot alkaloids, it is coupled with dimethylaminobenzaldehyde.[46] A solution of ergotamine in acetic acid gives a blue color with sulfuric acid, suitable for estimation.[47]

Procedure—*By p-dimethylaminobenzaldehyde.* Mix equal volumes of a 0.05 per cent solution of sodium nitrite and a 2 per cent solution of *p*-dimethylaminobenzaldehyde in 1:1 ethanol and concentrated hydrochloric acid. Mix a sample containing 0.1-1 mg. of ergotamine with 0.3 ml. of this reagent and follow with 0.6 ml. of concentrated sulfuric acid. After 5 minutes, dilute with water to 20 ml. Read at 530 mμ against a reagent blank.

By acetic and sulfuric acids. Measure 1 ml. of sample, diluted to 0.05-0.5 mg. of ergotamine per ml. calculated as tartrate. Render the sample distinctly alkaline with 1:1 ammonium hydroxide. Extract with 2, 2, and 1 ml. of ether. Combine the ether extracts and evaporate to dryness. Dissolve the residue in 2 ml. of glacial acetic acid. Add 2 ml. of concentrated sulfuric acid. Mix and read against a reagent blank.

Ergotoxine

Ergotoxine is determined by the general reaction of ergot alkaloids with *p*-dimethylaminobenzaldehyde.[48] Either it is separated and determined, or the water-soluble fraction is separately determined and subtracted also to give ergometrine by difference.[49]

Sample—*Ergotoxine-ergometrine mixtures.* Mix 20 ml. of the aqueous sample with 30 ml. of a buffer for pH 4 (Vol. I, page 176). Extract

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[46] Lawrence S. Malowan, *Ciencia* (Mexico) **9**, 124-5 (1948).

[47] Ole Bartsch, *Dansk Tids. Farm.* **5**, 172-5 (1931).

[48] Svend Aage Schon and I. Bennekou, *Ibid.* **12**, 257-85 (1938).

[49] C. K. Glycart, *J. Assoc. Official Agr. Chem.* **20**, 566-8 (1937); *Ibid.* **21**, 538-41 (1938).

in a continuous liquid-liquid extractor for 4 hours with ether. Separate the ether solution which contains all of the ergotoxine and dilute to 100 ml. Reserve the aqueous layer for isolation of the ergometrine. Extract the ether solution successively with six 10-ml. portions of 1 per cent tartaric acid solution. Heat the combined extracts to drive off ether and dilute to a known volume containing 0.005-0.02 mg. of ergotoxine per ml. Use the direct method.

Fluid extract of ergot. Evaporate a 20-ml. sample to about 5 ml. and take up with 15 ml. of water. Add 30 ml. of a buffer for pH 4 and extract in a continuous liquid-liquid extractor for 4 hours. Separate the ether extract containing ergotoxine and pigments. Save the aqueous layer for determination of ergometrine. Shake the ether layer with a phosphate buffer for pH 9.2 until pigments are removed. Complete as for other mixtures, starting at "Extract the ether solution successively . . . ."

Ergot. Defat the ergot with petroleum ether. Make 10 grams faintly alkaline with 1:10 ammonium hydroxide and extract in a continuous extractor with ether. Dilute to 100 ml. For the sample for total alkaloids extract 50 ml. of ether solution by the method for ergotoxine-ergometrine mixtures, starting at "Extract the ether solution successively . . . ." For the sample for water-soluble alkaloids, extract the other 50 ml. of ether solution with water in a continuous liquid-liquid extractor for 4 hours. Dilute this extract to 50 ml. and make it 1 per cent with tartaric acid.

Procedure—*Direct.* Develop 1 ml. of the sample with *p*-dimethylaminobenzaldehyde as described for ergot alkaloids (page 447).

Indirect. Develop 1 ml. of the sample containing the total alkaloids, reading as ergotoxine. Develop 1 ml. of the water-soluble alkaloid fraction, reading as ergotoxine. In each case use *p*-dimethylaminobenzaldehyde as reagent. The second value is ergotoxine. The difference multiplied by 1.86 is ergometrine.

β-Erythroidine

β-Erythroidine, an alkaloid of curare-like physiological action, is transformed by strong sulfuric acid to apo-β-erythroidine which gives an intense purple color with ferric chloride solution.[50] The color is

[50] Emma M. Dietz and Karl Folkers, *J. Am. Pharm. Assn.* 35, 489 (1946).

stable for 24 hours at room temperature. The other erythrine alkaloids give slight color in this reaction. Erysodine, erysovine, erysopine, erythraline, and erythratine develop varying amounts of brown, light-absorbing substances. However, the absorption at the wave length used is only one-fourth that of β-erythroidine. Therefore, a 10 per cent contamination with these alkaloids results in +3 per cent error.

Procedure—To 5 ml. of solution containing 0.6 mg. of alkaloid, slowly add 15 ml. of 2:1 sulfuric acid. Heat in an oil bath for 1 hour at 115°. Add 0.3 ml. of 10 per cent ferric chloride solution and shake. Heat for 8 minutes at 115° to develop the color. Cool and dilute to 40 ml. with 2:1 sulfuric acid and read at 540 mμ against a reagent blank.

Diacetylmorphine, Heroin

A method for development of color with iodic acid and a ferric salt is described under morphine (page 459). Alternatively, hydrolyze with acid and determine as nitrosomorphine in alkaline solution (page 459).

Homatropine Methylbromide, Novatropine

Homatropine methylbromide is quantitatively precipitated from aqueous solutions with ammonium reineckate. The precipitate is then dissolved in acetone and determined at 525 mμ.[51] For details of a method with bromocresol purple see atropine (page 430).

Sample—*Tablets.* Grind 20 or more tablets representing 5 mg. of homatropine methylbromide to a fine powder. Add 5 ml. of water and allow to stand for 30 minutes, shaking at intervals. Filter through a medium-porosity fritted-glass crucible and wash with five 1-ml. portions of water.

Procedure—Add 2 grams of ammonium reineckate to 100 ml. of water and stir mechanically for 10 minutes. Filter and keep under refrigeration. Add 1 ml. of 1:4 sulfuric acid to the combined filtrate and washings. Add 2 ml. of ammonium reineckate reagent to the filtrate and washings. Allow to stand for 1 hour to complete precipitation and then filter. Wash the precipitate with two 2-ml. portions of water and apply suction to remove all water. Dissolve the precipitate by passing 2-ml. portions of acetone through the crucible with the aid of gentle

[51] F. J. Bandelin, *Ibid.* 37, 10-12 (1948).

suction. After 4 portions have been passed through, adjust the volume to 10 ml. with acetone. Read at 525 mμ against acetone.

p-(2-Dimethylaminoethyl)phenol, Hordenine

Extract the hordenine from malt with diethylether. Diazotize and couple with *p*-aminobenzenesulfonamide to get a pale-red compound which is read colorimetrically.[52]

Hydrastine

Hydrastine is determined, after oxidation to hydrastinine and opianic acid, by measurement of the fluorescence of hydrastinine.[53] Canadine and berberine, also present in *Hydrastis canadensis*, do not interfere after extraction with ether. The method is accurate to about ± 1 per cent. Alternatively determine as the reineckate as described under alkaloids in general (page 428).

Sample—*Crude hydrastis.* Shake 0.4 gram of finely powdered hydrastis with 50 ml. of peroxide-free ether for 2 minutes. Add 1 ml. of 1:1.6 ammonium hydroxide and shake mechanically for 30 minutes. Warm 25 ml. of the ether extract to remove nearly all the ether. Add 10 ml. of fresh ether and evaporate to about 2 ml. Add 10-15 ml. of 1:360 sulfuric acid and drive off all the ether. Cool and dilute to 100 ml. with a 1:360 sulfuric acid.

Procedure—Oxidize 5 ml. of the prepared sample solution with 10 ml. of 3:1 nitric acid, by warming at 50° for a half-hour. Cool and dilute to 100 ml. with water at 20°. Adjust the fluorometer to a reading of 50 with a solution containing 3.6×10^{-4} mg. per ml. of quinine sulfate in 1:360 sulfuric acid, using Coleman B-1 primary filter and PC-2 secondary filter. Dilute 10 ml. of sample solution to 100 ml. with water and measure the fluorescence.

Hydroxydihydrocodeinone

The reaction is the same as described for dihydrocodeinone (page 442).

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52 Mario Pedinélli, *Ann. chim. applicata* **31**, 410-4 (1941).

53 Einar Brochmann Hanssen and Joseph A. Evers, *J. Am. Pharm. Assoc.* **40**, 620-22 (1951).

Hyoscyamine

Methods of determination are the same as for the optically inactive form known as atropine. Details are given under atropine (page 429).

Lobeline

Lobeline is split to give actophenone at pH 6-10 and then estimated as the derivative.[54]

Sample—Mix an aqueous solution containing 1-2.5 mg. of lobeline with 20 ml. of buffer solution for pH 8 and dilute to 70 ml. with water. Reflux for 15 minutes and then distil at 3 ml. per minute to collect 30 ml. Dilute to 100 ml. with water and develop a 5-ml. aliquot.

Procedure—Determine acetophenone by 2,4-dinitrophenylhydrazine (Vol. III, page 301).

Lupanine

Lupanine is precipitated by silicomolybdic acid and the precipitate reduced to molybdenum blue.[55]

Sample—*Lupine meal.* Extract the lupanine from a 15-gram sample by shaking frequently during a 24-hour period with 100 ml. of ether, 50 ml. of chloroform, and 10 ml. of 15 per cent sodium hydroxide solution. If the upper layer is not clear, add a few drops of water and shake. Filter through a fluted filter. To a 50-ml. aliquot of filtrate, add 50 ml. of ether, and shake with three 20-ml. portions of 1:35 hydrochloric acid. Draw off the acid solution as completely as possible each time. Heat these acid solutions gently to drive off traces of ether and chloroform.

Procedure—Develop by the procedure given at Vol. III, page 368.

Cannabis, Marihuana

The active portion of marihuana resin is predominantly phenolic. The main inactive phenol is not soluble in dilute sodium hydroxide

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[54] Kerstin Matérn and Göran Schill, *Svensk Farm. Tids.* **54**, 445-50 (1950).

[55] Karl Meyer, *Landw. Jahrb.* **91**, 418-40 (1941); F. Machad P. Lederle, *Landw. Vers. Sta.* **98**, 117-24 (1921).

solution. This is the basis for the separation for determination by diazotized *p*-nitroaniline.[56]

Sample—Extract an appropriate sample, according to source, with 50-75 ml. of carbon tetrachloride in a Soxhlet. Shake the extract with four 20-ml. portions of 0.5 per cent sodium hydroxide solution. Dilute the extracts to 100 ml. and mix.

Procedure—Dissolve 0.2 gram of *p*-nitroaniline in 5 ml. of 1:1 hydrochloric acid and cool in ice. Add 5 ml. of cold 2 per cent sodium nitrite solution, keeping the temperature at 0° Add the diazotized solution to 10 ml. of the extract. A red dye will separate out, leaving a yellow solution. Saturate the liquid with sodium chloride to complete precipitation, filter, and suck dry. Dissolve the dye in 10 ml. of ethanol and dilute to 25 ml. with ethanol. Read against ethanol as the blank.

3,4,5-Trimethoxyphenethlamine, Mescaline

In the absence of interfering substances, mescaline is read by the color with picric acid as extracted into 1:1 chloroform-toluene. Follow the details for benzedrine (page 51) and read at 420 mμ. Benzedrine under the same circumstances absorbs at 410 mμ. The yellow color in chloroform from formation of a quininoid structure with bromocresol purple is read at 410 mμ.[57] Other solvents and sulfonaphthaleins are appropriate. Cocaine gives the same reaction.

Morphine

After morphine is oxidized in acid solution with iodic acid a violet color develops with a very dilute solution of ferric chloride,[58] ferrous chloride,[59] or nickel sulfate.[60] Excess of iron interferes by precipitation

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[56] H. B. Dunnicliff, *Analyst* **68**, 70-4 (1943).

[57] L. A. Woods, J. Cochin, E. J. Fornefeld, F. G. McMahon, and M. H. Seevers, *J. Pharmacol. Exptl. Terap.* **101**, 188-99 (1951).

[58] Otto Folin and H. Malmros, *J. Biol. Chem.* **83**, 115-20 (1929); Salvatore Guarino, *Boll. soc. ital. biol. sper.* **20**, 754-7 (1945); *Ibid.* **22**, 253-5 (1946); Laura Nicolini, *Ann. pharm. franc.* **5**, 528-34 (1947); Aurelio Mariani, Salvatore Guarino, and Olga Mariani Marelli, *Ann. chim.* **41**, 661-8 (1951); *Rend. ist. super. sanità* (Rome) **14**, 733-41 (1951); C. F. Moorhoff, *Pharm. Weekblad.*, **87**, 593-601 (1952).

[59] B. Drevon and G. Lafitte, *Ann. pharm. franç.* **8**, 397-401 (1950).

[60] J. S. N. Crames and J. G. Voerman, *Pharm. Weekblad.* **84**, 129-33 (1949); *Acta Pharm. Intern.* **1**, 219-24 (1950).

as ferric iodate or hydroxide. The same reagent is applicable to estimation of both morphine and heroin in mixtures.[61] The heroin does not react with iodic acid so that the morphine is determined directly in the mixture. Then heroin is converted to morphine by saponifying with 2.75 per cent sulfuric acid at 100° for 50 minutes in a closed tube and the sum is determined by the same method. Traces of tannins give the reaction and therefore interfere.[62] An analogous reaction is obtained with hydrogen peroxide and copper sulfate.[63] The color is red in the presence of ammonium hydroxide.

Morphine can be converted to nitrosomorphine and determined by its orange color in alkaline solution.[64] Codeine, narceine, papaverine, and thebaine do not interfere. The method is accurate to about 4 per cent for 1-5 mg. of morphine hydrochloride.

Advantage is taken of the phenolic group in morphine for its estimation by phosphomolybdic acid.[65] The color is blue as is usual in such methods. Separation from other substances giving the phenolic reaction is necessary. Codeine, dionin, heroin, and peronin do not interfere. Replacement of ammonium hydroxide by 10 per cent sodium carbonate results in a more stable color.[66] Similarly, morphine reduces silicomolybdic acid in alkaline solution to a molybdenum blue.[67] Morphine must be quantitatively isolated from reducing substances. The method is accurate to about ±2 per cent. Fading is often a problem.[68]

The free phenolic group of morphine reacts with ammonium iodoxy-

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[61] Ryoichi Ito, *Manshu, Ig. Z.* **24**, 1-44 (1936).

[62] Virgilio Lucas, *Rev. brasil. farm.*, **31**, 229-30 (1950).

[63] Georges Deniges, *Bull. soc. pharm. Bordeaux* **50**, 465-8 (1910); L. Magendie, *Ibid.* **65**, 157-78 (1927); F. Bamford, *Analyst* **56**, 586-9 (1931); Joseph B. Oram, *Am. J. Pharm.* **122**, 60-6 (1950).

[64] Heinrich Wieland and Paul Kappelmeier, *Ann.* **382**, 306-39 (1911); G. Ya. Khait., *Ukrain. Gosudarst. Inst. Eksptl. Farm.* (Kharkov), *Konsul'tatsionnye Materialy* **1939**, No. 3, 71-2; S. M. Bolotnikov, *Ibid.* **1939**, No. 6, 169-70; D. C. M. Adamson and F. P. Handisyde, *Analyst* **70**, 305-6 (1945); *Pharm. J.* **157**, 104 (1946); A. Denoël, *J. pharm. Belg.*, [N.S.] **1**, 241-7 (1946); Anders Baerheim Svendsen, *Dansk Tids. Farm.* **22**, 131-60 (1948); W. Poethke, *Pharm. Zentralhalle* **90**, 145-51 (1951); R. L. Stevens, *J. Pharm. Pharmacol.* **3**, 315-22 (1951).

[65] Juan A. Sanchez, *Semana Méd.* (*Buenos Aires*) **37**, 333-4 (1930); *Ibid.* **42**, 191-5 (1935); *J. pharm. chim.* **21**, 366-76 (1935).

[66] Ramón San Martín Casamada and Arturo Mosqueira Toribio, *Anales real acad. farm.* **15**, 343-8 (1949); *Ibid.* **14**, 433-44 (1948).

[67] R. Hofmann and N. Popovici, *Pharm. Zentralhalle* **76**, 346-8 (1935); Z. M. Vaĭsberg, Ya. A. Fialkov, and E. G. Khrizman, *Farmatsiya* **9**, No. 4, 18-26 (1946).

[68] C. G. van Arkel, *Pharm. Weekblad* **74**, 134-7 (1937).

benzoate to give an orange color suitable for estimation.[69] The same color is developed by slow spontaneous oxidation of morphine solutions in air. Sucrose and lactose do not interfere. The negative ion combined with the morphine has no effect. Codeine, heroin, and dionin do not react. Heavy metals precipitate the reagent. Reasonable excess of the reagent has no effect other than quicker development of the maximum color; excessive amounts remain as a precipitate. The color is substantially permanent. Accuracy to 3 per cent is obtainable. An alternative is coupling with diazotized amines.[70]

Morphine is determined nephelometrically as a morphine-vanadium-molybdic complex formed with vanadomolybdic acid.[71] The complex is probably $V(OH)_5 \cdot 2MoO_3 \cdot C_{17}H_{19}NO_3$. Other alkaloids than morphine, heroine, and dilaudid are precipitated by molybdic acid and filtered. The two mentioned may interfere. The amount of morphine determined must not exceed 0.2 mg. In the handling of samples with a very low morphine content, such as 0.001 mg. or less per ml., loss may occur by oxidation unless pyrogallol is added as a protective agent.[72] The determination is made more specific by extraction of urine by ether before and after making alkaline with sodium bicarbonate.[73]

Samples—*Laudanum.* Add 5 ml. of methanol to 1 ml. of laudanum and 1 gram of alumina and shake 2-3 minutes to decolorize. Filter and wash twice with 5 ml. of methanol. Use the entire filtrate for development by ferric chloride.

For development by phosphomlybdic or silicomolybdic acid, digest 0.1 gram of laudanum with 3 ml. of saturated lime water for 1 minute. Cool and filter. Dilute to 500 ml. for use of an aliquot.

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69 Chauncey D. Leake, *Proc. Soc. Exptl. Biol. Med.* 28, 148-50 (1930); George A. Emerson, *Ibid.* 31, 1004-6 (1934); C. Robert Moodey and G. A. Emerson, *Univ. Calif. Pub. Pharmacol.* 1, 235-47 (1939).

70 E. I. Ginzburg and N. I. Gavrilov, *Zhur. Anal. Khim.* 1, 282-4 (1946); F. Balák and A. Jindra, *Casopis Ceského Lékárnictva* 63, 125-36 (1950); Kikuo Asahina, *Ann. Rept. Takeda Research Lab.* 10, 69-73 (1951).

71 W. Deckert, *Arch. exptl. Path. Pharmakol.* 180, 656-7 (1936); *Z. anal. Chem.* 112, 241-57 (1938); Mutuzi Endō, *Hokkaido Igaku Zasshi* 16, 1166-70 (1938); Mutuzi Endō and Tosio Katō, *Proc. Japan. Pharmacol. Soc.* 12, 136-8 (1938); Fred W. Oberst, *J. Lab. Clin. Med.* 24, 318-29 (1938); M. Schirm, *Deut. Apoth. Ztg.* 55, 106-7 (1940); Wolfgang Arnold, *Deut. Gesundheitsw.*, 7, 946-50 (1952).

72 Charles E. Morgan, *Ind. Eng. Chem., Anal. Ed.* 9, 383-4 (1937).

73 P. Seifert and G. Jankowitz, *Arch. exptl. Path. Pharmakol.* 214, 197-201 (1952).

Opium. Triturate 0.5 gram of opium with a few ml. of water. Add gram of calcium hydroxide, suspend in 10-15 ml. of water after 15 minutes, and shake frequently during a 15-minute period. Add 10-20 ml. of water, centrifuge, and filter the supernatant liquid. Suspend the solid in 20-30 ml. of water and repeat this extraction 3 times. Combine the extracts and dilute to 500 ml. with water. Develop an appropriate aliquot with ferrous chloride or copper sulfate.

For development by sodium nitrite, triturate 0.05 gram of opium with 0.1 ml. of glacial acetic acid. Add 5 ml. of water and filter after 5 minutes. Add 2 ml. of 5 per cent mercuric chloride solution to the yellow filtrate, then heat at 90° for 2 minutes. Cool in ice for 10 minutes and filter. Add 0.1 gram of calcium hydroxide to the filtrate, mix, and add 1 ml. of 10 per cent sodium hydroxide solution. Filter again and add 0.5 gram of ammonium sulfate. Extract the free morphine thrice with 35-ml. portions of a 4:1 mixture of chloroform and isopropanol. Extract the combined extracts thrice with 10-ml. portions of 0.4 per cent sodium hydroxide solution. Neutralize the aqueous extract and add 3.5 ml. of 1:5 hydrochloric acid.

For development by phosphomolybdic or silicomolybdic acid, mix 0.01 gram of finely powdered opium with 3 ml. of saturated lime water and heat at 100° for 2 minutes. Cool, filter, and wash the filter with two 2-ml. portions of water. Acidify with 2 drops of 1:5 hydrochloric acid and shake. Add 10 ml. of a 4:1 mixture of chloroform and isopropanol. Make alkaline with 2 drops of 1:2 ammonium hydroxide and shake. Separate the solvent layer, extract the aqueous layer with 5 ml. of the solvent, and filter the extracts. Evaporate the extracts at 100°. Take up the residue with 10 ml. of 1:5 hydrochloric acid and filter. If the opium contained 10 per cent of morphine, 1 ml. will be a suitable sample.

Ampoules. Dilute 1 ml. to 100 ml. and mix. Develop by phosphomolybdic acid.

Syrup. Dilute 2 grams of sirup to 10 ml. and develop by phosphomolybdic acid.

Capsules.[74] Grind a 2-gram sample with 2 ml. of 10 per cent sodium carbonate solution. After 1 hour add 2 ml. of 20 per cent ammonium chloride solution and shake with 20 ml. of 1:1 benzene-butanol. Separate the solvent and extract with 20 ml. of 1:1.4 sulfuric acid. Neutralize to a faint acidity, dilute to 50 ml., and develop an aliquot by nitrite.

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[74] J. F. Reith and A. W. M. Indemans, *Pharm. Weekblad* **85**, 309-19 (1950).

Opium extract. Dissolve 0.2 gram of opium extract in 20 ml. of water at not over 70-80°. When solution is complete, cool with water. Grind 2 grams of hydrated calcium oxide and add the solution of sample slowly, with mixing. Filter after grinding for 4-5 minutes and develop by copper sulfate.

Tincture of opium. Grind 0.6 gram of hydrated lime in a mortar. Add 6 ml. of tincture of opium drop by drop. Continue to mix for 2-3 minutes. Filter and wash well with water. Dilute to 500 ml. for development of an aliquot by phosphomolybdic or silicomolybdic acid.

Paregoric. Because of the camphor and essence of anise present, lime will not decolorize this. Shake 15 ml. of sample and 15 ml. of water. A white emulsion is obtained. Add 15 ml. of ether and mix 3-4 minutes by reversing rather than shaking the tube, to avoid further emulsification. Let stand and, when 2 layers separate, discard the supernatant ether. Repeat the extraction with 15 ml. more of ether. If the liquid is still cloudy, repeat. After removal of the ether dilute the aqueous layer to 30 ml. and develop by copper sulfate.

Morphine sirup. Mix 10 ml. of morphine sirup and 10 ml. of water for development by copper sulfate.

Poppy plants.[75] Disintegrate about 0.4 gram and heat with 8 ml. of hot 3 per cent barium hydroxide solution. Filter and repeat. Add acetic acid to an aliquot of the filtrates and develop by ferric chloride.

Dover powder.[76] Add 1:10 hydrochloric acid to a 1-gram sample until neutral and dilute to about 10 ml. Heat to boiling with frequent shaking for about an hour and cool. Dilute to about the original volume and filter. Evaporate the filtrate to about 2-2.5 ml. and add 4 ml. of 1.355 per cent mercuric chloride solution in 5 per cent aqueous potassium iodide. Mix and after 2 minutes filter and wash on the filter. Dissolve the precipitate in 10 ml. of 0.4 per cent sodium hydroxide solution and filter. Develop the filtrate by sodium nitrite.

Urine.[77] Make 20 ml. of urine acid to litmus with 1:10 acetic acid and triturate with 35 grams of anhydrous sodium sulfate. In an extraction tube on a cotton base, put 0.2 gram of activated carbon, 0.2 gram of magnesium oxide, and 2 grams of anhydrous sodium sulfate, and

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[75] A. Ginzberg and N. Krashevskii, *Org. Chem. Ind.* (USSR) 2, 104-7 (1936).

[76] G. Ya. Khait, *Ukrain. Gosudarst. Inst. Eksptl. Farm.* (Kharkov), *Konsul'tatsionnye materialy* **1940**, No. 5, 159-62.

[77] H. Griffon and R. LeBreton, *J. pharm. chim.* **28**, 49-60 (1938); Salvatore Guarino, *Boll. soc. ital. biol. sper.* **22**, 1226-8 (1946).

the sample. Percolate slowly with 30 ml. of ether and evaporate from the receiver. Take up the residue in 5 ml. of methanol and develop by ferric chloride.

For nephelometric development, heat 10 ml. of urine to boiling with 0.3 gram of sodium bicarbonate, and cool. Shake for 0.5 minute with 20 ml. of ethyl acetate. Evaporate the ethyl acetate layer and dissolve the residue in 0.25 ml. of water containing 1 drop of concentrated nitric acid and 1 drop of 10 per cent ammonium molybdate solution. Filter through a cotton pledget and rinse the evaporating dish first with 0.25 ml. of water and then with 0.15 ml.

Blood.[78] Precipitate 10 ml. of blood with 40 ml. of ethanol and evaporate the filtrate. Dissolve the residue in 10 ml. of water containing 2 drops of concentrated nitric acid and make alkaline with 0.3-0.5 gram of sodium bicarbonate. Continue as for nephelometric development of urine, starting at "Shake for 0.5 minute with 20 ml. of ethyl acetate."

Procedure—*By iodic acid and ferric chloride.* To the sample solution add 15 ml. of 1:120 hydrochloric acid and 2 ml. of 5 per cent iodic acid solution. After 2 minutes add 5 ml. of saturated ammonium carbonate solution. When an intense orange-yellow color develops, dilute to 50 ml. with 5 per cent ammonium carbonate solution. Add 1 ml. of 1 per cent ferric chloride solution and read at 430-590 mμ against a reagent blank.

By iodic acid and ferrous chloride. To an appropriate sample, add 1 ml. of 1:10 hydrochloric acid, 1 ml. of 10 per cent iodic acid, and after 1 minute 1 ml. of cold saturated ammonium carbonate solution. Then add 3 drops of freshly prepared solution of 1:1000 ferrous chloride or 1.7:1000 ferrous sulfate. Read at 470 mμ against a reagent blank.

By copper sulfate. The sample will ordinarily be a lime extraction. Make it just acid to litmus with 1:1 hydrochloric acid and dilute to a known volume for use of aliquots. To 10 ml. of sample add 1 ml. of 3 per cent hydrogen peroxide and 1.5 ml. of concentrated ammonium hydroxide. Invert 3 or 4 times to mix. Add 1 drop of a solution prepared by mixing 50 ml. of 3 per cent copper sulfate pentahydrate solution and 60 ml. of concentrated hydrochloric acid. Read after about 10 minutes against a sample blank.

By sodium nitrite. Neutralize a sample containing about 5 mg. of morphine, if alkaline, and make 1:120 with hydrochloric acid. Dilute

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[78] W. Deckert, *Arch. exptl. Path. Pharmakol.* **180**, 656-7 (1936).

to about 20 ml., add 8 ml. of 1 per cent sodium nitrite solution and mix well. After 15 minutes, add 12 ml. of 1:10 ammonium hydroxide, dilute to 50 ml., and mix again. Read at 430 mμ against a sample blank.[79] Alternatively replace the ammonium hydroxide with 5 ml. of 30 per cent sodium hydroxide solution and read at 470 mμ.

By phosphomolybdic acid. As reagent dissolve 140 grams of sodium carbonate and 20 grams of disodium phosphate in 500 ml. of water. Add 70 grams of recently dried molybdic acid and 200 grams of concentrated nitric acid. Dilute to 1 liter and stir frequently. After 24 hours, filter.

To 10 ml. of sample solution add 1 ml. of reagent and 1 drop of concentrated nitric acid. Let stand for 10 minutes, add 1 ml. of concentrated ammonium hydroxide, and mix. The maximum color will develop in 1 minute and persist for 15 minutes. Read against a reagent blank.

By silicomolybdic acid. As reagent dissolve 14.4 grams of molybdic anhydride in 100 ml. of 4 per cent sodium hydroxide solution by warming. Add a sodium silicate solution of known concentration until 0.7 gram of silica is present. Add 1:3 hydrochloric acid in small portions with stirring until the solution becomes green. This usually requires about 200 ml. Dilute to about 900 ml. with water and leave for 3 hours on the water bath. Remove and let stand to clarify. After 24 hours, filter off excess silicic acid and dilute the filtrate to 1 liter.

To the prepared sample add 2 ml. of the silicomolybdic acid reagent and 5 ml. of 1:6 ammonium hydroxide. Take care of any undue acidity of sample and standard by corresponding increase in the amount of ammonium hydroxide added. Dilute to 25 ml. with water and read after 5 minutes against a sample blank.

By ammonium iodoxybenzoate. To 5 ml. of sample solution add about 50 mg. of powdered ammonium iodoxybenzoate and mix. Read after 30 minutes against a sample blank.

Nephelometrically as vanadium-molybdate complex. To the prepared solution, add 2 drops of 2 per cent ammonium vanadate solution. A gradual opalescence appears. After 20 minutes, dilute to 5 ml. and read nephelometrically.

β-[N-(3-Hydroxy-4-pyridone)]-α-aminopropionic acid, Mimosine

Mimosine is developed by ferric chloride in acid solution.[80]

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[79] D. C. Garratt, *Quart. J. Pharm. Pharmacol.* 10, 466-70 (1937).

[80] Hiromu Matsumoto and G. Donald Sherman, *Arch. Biochem. Biophys.* 33, 195-200 (1951).

Procedure—Digest the sample with an appropriate amount of 1:100 hydrochloric acid and let cool. Filter or centrifuge and treat 10 ml. of extract with 30 mg. of activated carbon. Filter, wash, and add 4 ml. of 0.5 per cent ferric chloride solution in 1:100 hydrochloric acid. Dilute to 100 ml. with 1:100 hydrochloric acid and read against a reagent blank.

NARCEINE

Narceine is determined by the color on oxidation with bromine [81] with accuracy to ± 3 per cent.

Procedure—Dilute a sample containing about 1 mg. of narceine to about 7 ml. Add 0.5 ml. of 1:120 hydrochloric acid and 0.5 ml. of saturated bromine water. After 5 minutes add 1.5 ml. of 6.4 per cent aqueous sodium sulfite and incubate at 50° for 3 minutes. Develop the color with 0.2 ml. of 1:10 ammonium hydroxide and read against a reagent blank.

NARCOTINE

Narcotine may be coupled with diazotized sulfanilic acid after nitric acid treatment.[82] Codeine and thebaine interfere.

Procedure—Make the solution of sample 1:20 with concentrated nitric acid and heat for 15 minutes in boiling water. Cool, couple with diazotized sulfanilic acid (page 419), and read after 1-1.5 minutes at 500 mμ against a reagent blank.

1-METHYL-2-(3-PYRIDYL)PYRROLIDINE, β-PYRIDYL-α-N-METHYLPYROLIDINE, NICOTINE

Aside from nicotine as such, nornicotine is present in many cases in variable amount. In some tobaccos nornicotine predominates. Both the pharmacological and toxicological properties of the two differ. Therefore, some methods are applied to determination of nicotine, and others for the sum of nicotine and nornicotine.

When cyanogen bromide is added to a dilute solution of nicotine, a pale yellowish green develops. The corresponding reaction with nor-

[81] Z. M. Vaĭsberg, Ya. A. Failkov, and E. G. Khrizman, *Farmatsiya* **9**, No. 4, 18-26 (1946); *Ibid.* **10**, No. 1, 26-30 (1947).

[82] Erich Wegner, *Pharmazie* **5**, 83-5 (1950).

nicotine results in red.[83] Each may be read separately. Potassium dihydrogen phosphate is added to the reaction mixture to separate the maxima of the color of nicotine and nornicotine and to increase their color stability. Inorganic salts markedly influence the rate of development of color and increase its maximum intensity. Other salts slow the rate of color development, increase stability, and increase or decrease the maximum intensity of color. As to nornicotine, phosphate ion, ammonium ion, or molybdate ion alters the transmittance curves. Sodium chloride, potassium chloride, and sodium sulfate produce the same effect in saturated solutions but permit color to fade rapidly.

Addition of an amine converts the yellowish-green of nicotine to pink or red so that the total of nicotine and nornicotine is read. Typical amines for the purpose are aniline,[84] β-naphthylamine,[85] and benzidine.[86]

Another reaction involves the formation of a ketone group on the nicotine.[87] A red color develops upon addition of sodium nitroprusside and ammonium hydroxide to a solution of nicotine. Coniine and sparteine interfere, but pyridine does not. Accuracy is to ±2 per cent.

Nicotine is determined indirectly by precipitation with silicomolybdic acid and reduction of the latter to a blue compound.[88] The same blue compound is formed by the direct reduction of silicomolybdic acid as by the reduction of its complex with the alkaloid. The blue color from silicomolybdic acid is more stable than that from phosphomolybdic acid. The method is accurate to 2-3 per cent.

Adrenaline, morphine, curare, hydroquinone, cresol, vitamin B_1, and thymol give the same color. Atropine, cocaine, piperidine, procaine, quinidine, and veratrin give a turbid solution. There is no reaction by

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[83] P. S. Larson and H. B. Haag, *Ind. Eng. Chem., Anal. Ed.* 16, 88 (1944).

[84] A. S. Borozdina, *Vsesoyuz. Inst. Tabach. i Makhoroch. Prom.* No. 133, 158-65 (1937); E. Werle and H. N. Becker, *Biochem. Z.* 313, 182-91 (1942).

[85] László Barta and Zoltán Marscheck, *Mezögazdasági Kutatások* 10, 29-35 (1937); L. N. Markwood, *J. Assoc. Official Agr. Chem.* 22, 427-36 (1939); *Ibid.* 23, 792-800 (1940); *Ibid.* 23, 804-10 (1940); William E. McCormick and Maxine Smith, *Ind. Eng. Chem., Anal Ed.* 18, 508-12 (1946); A. R. Trim, *Biochem. J.* 43, 57-60 (1948); William A. Wolff, Marina A. Hawkins and W. E. Giles, *J. Biol. Chem.* 175, 825-31 (1948).

[86] A. C. Corcoran, O. M. Helmer and Irvine H. Page, *Ibid.* 129, 89-97 (1939).

[87] Luis Policarpo, *Anales farm. bioquím.* (Buenos Aires) 12, 117-41 (1941).

[88] Rudolf Hofmann, *Biochem. Z.* 260, 26-33 (1933); G. L. Sutherland, R. P. Daroga, and A. G. Pollard, *J. Soc. Chem. Ind.* 58, 284-8 (1939); Michio Takeuchi and Kenichi Mikami, *J. Nara Med. Assoc.*, 3, 10-12 (1952); Giovanni Ghezzi *Ann. chim.* (Rome), 43, 48-54 (1953).

nicotinic acid, aniline, dextrine, phenolphthalein, pyridine, saponine, glucose, galactose, calcium levulinate, maltose, strychnine, picrotoxin, and xylose.

Another method is based on the formation of a colored complex salt of nicotine with bromothymol blue dye in the presence of an acid.[89] The colored salt is extracted with chloroform for reading to ±2 per cent for less than a milligram of nicotine.

The addition of a solution of silicotungstic acid to a dilute, neutral, or acidic nicotine solution produces a white turbidity which is used for nephelometric estimation [90] with a minimum solubility of 5.3 mg. per liter in 0.005 N hydrochloric acid. Nicotine is also determined nephelometrically by phosphotungstate.[91] Sulfuric acid sensitizes the method, and the warming and cooling technic is carried over from the silicotungstate procedure. Organic bases and alkaloids, proteins, amino acids, lead, acetone, and ether interfere. Contents of salts not otherwise interfering must be standardized.

Nicotine in acid solution can be read in the ultraviolet but is indistinguishable from nornicotine.[92] Unless steam-distilled, there are many interferences by plant pigments, lipides, degradation products, etc. Practically by reading at 259 mμ and correction for the average background absorption at 236 mμ and 282 mμ, good values are obtained.

Samples—*Tobacco.* To 0.5-2 grams of finely ground tobacco add 10 grams of sodium chloride, 10 ml. of 40 per cent sodium hydroxide solution, and a little paraffin. Place in the receiver 3 ml. of 1:180 sulfuric acid and enough water to cover the exit of the condenser. Steam-distil to collect about 800 ml. in 1 hour, maintaining the volume in the flask from which distillation is being carried out at 50-75 ml. Adjust the pH of the distillate to 4.1-4.2 with 1:180 sulfuric acid or 1 per cent potassium hydroxide solution and dilute to 1 liter for development by cyanogen bromide. For determination by silicomolybdic acid use 1:35 hydrochloric acid throughout in place of 1:180 sulfuric acid.

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[89] C. Kinthakul and J. Hannen, *J. Soc. Chem. Ind.* **69**, 126-8 (1950).

[90] Tokurchiro Kozu, *J. Agr. Chem. Soc. Japan* **7**, 977-83 (1931); R. Sengbusch, *Der Suchter* **3**, 33-8 (1931); *Deut. Landw. Rundschau* **8**, 145 (1931); Joseph R. Spies, *J. Am. Chem. Soc.* **58**, 2386-8 (1936).

[91] L. N. Markwood, *J. Assoc. Official Agr. Chem.* **23**, 800-10 (1940).

[92] Margaret L. Swain, Abner Eisner, C. F. Woodward, and B. A. Brice, *J. Am. Chem. Soc.* **71**, 1341-5 (1949); C. O. Willits, Margaret L. Swain, J. A. Connelly, and B. A. Brice, *Anal. Chem.* **22**, 430-3 (1950).

By extraction. To about 1 gram of tobacco add 1 ml. of 20 per cent sodium hydroxide solution and 20 ml. of light petroleum ether. Shake for 2 hours, remove 5 ml. of the petroleum ether extract, and shake with 20 ml. of 1:1200 hydrochloric acid. Remove the aqueous layer. Wash the petroleum ether layer with 10 ml. of water and add this washing to the aqueous layer just removed. Dilute the aqueous extract to 50 ml. and use a 1-2 ml. aliquot for development by cyanogen bromide.

Fresh tobacco leaf. Disintegrate a weighed portion of fresh tobacco leaf by moistening with 6 drops of 90 per cent sulfuric acid. Dilute to about 30 ml. and add 1.2 grams of lead monoxide. Shake for a couple of minutes to coagulate and dilute to 100 ml. Mix and filter. Add 0.25 gram of finely divided magnesium to the filtrate to displace lead and shake for 1 minute. Filter and determine by β-naphthylamine and cyanogen bromide.

Soil. Suspend soil containing about 1.5 mg. of nicotine in 200 ml. of water. Add 3 grams of lime and 20 grams of sodium chloride. Steam-distil, collecting 250 ml. of distillate in a receiver with 10 ml. of 1:35 hydrochloric acid. Evaporate the distillate to 5-10 ml. and dilute to 15 ml. with 1:35 hydrochloric acid. Develop by silicomolybdic acid.

Air. Nicotine can be present in air in the form of vapor itself or as tobacco dust. This method determines both. Draw air through an impinger (Vol. III, page 64) at a rate of 1 cubic foot per minute until the quantity of nicotine collected is at least 0.05 gram. Add enough water to the impinger to bring the volume to 100 ml., shake, and remove a 10 ml. aliquot. If dust is present, add 0.1 ml. of 30 per cent sodium hydroxide solution, stopper, and shake occasionally for 15 minutes. If no tobacco dust is present, omit the preceding sentence. Add 2 drops of phenolphthalein indicator solution and neutralize with 1:20 acetic acid to the disappearance of pink color. Avoid excess acid. Centrifuge for at least 20 minutes. Develop color by β-naphthylamine and cyanogen bromide.

Dust. Treat 0.1 gram of 150-mesh dust with 10 ml. of 0.1 per cent sodium hydroxide solution. Shake occasionally for 10 minutes and centrifuge. Neutralize to phenolphthalein and develop an aliquot with β-naphthylamine and cyanogen bromide.

Alternatively, shake 0.5 gram of dust with 1.5 ml. of 20 per cent sodium hydroxide solution and 20 ml. of benzene for 10 minutes. Centrifuge and extract 10 ml. of the clear upper layer with 15 ml. and 15 ml. of 1:360 sulfuric acid for 2 minutes. Neutralize 10 ml. of the acid

extracts to methyl red with 0.4 per cent sodium hydroxide solution, and add this amount of alkali to another 10-ml. aliquot without indicator present. Develop this by aniline and cyanogen bromide.

Tissue. Distil a weighed sample of tissue suspended in 25 ml. of water containing 0.3 gram of magnesium oxide and 10.2 grams of sodium chloride until 8 ml. of distillate are collected. Develop with aniline and cyanogen bromide. Alternatively, steam-distil from aqueous suspension and develop by β-naphthylamine and cyanogen bromide.

Blood. Lake 30 ml. of oxalated blood with 90 ml. of water and add with stirring 30 ml. of 50 per cent trichloroacetic acid solution. Stir vigorously with a mechanical stirrer for 5 minutes and filter. To 50 ml. of filtrate add 20 grams of sodium chloride, 10 grams of sodium hydroxide, and 5 ml. of ethanol. Steam-distil 20 ml. as sample. Develop by β-naphthylamine and cyanogen bromide.

Urine. Render a sample of urine containing about 0.2 mg. of nicotine alkaline to phenolphthalein with 40 per cent sodium hydroxide solution. Distil to a volume of 5-10 ml., collecting the distillate under the surface of 20 ml. of 1:10 hydrochloric acid. Concentrate the distillate to 5-10 ml., cool, and make alkaline to phenol red with 4 per cent sodium hydroxide solution. Extract with 40, 40, and 40 ml. of ether. Add 5 ml. of 1:10 hydrochloric acid and evaporate the ether on a steam bath. Add 8 drops of 0.02 per cent *o*-cresolphthalein to the residue and then treat it with 0.4 per cent sodium hydroxide solution and 1:120 hydrochloric acid until the color has disappeared at approximately pH 8.2. Add water and 12 per cent sodium acetate solution to yield a concentration of about 3 per cent sodium acetate. Develop by benzidine and cyanogen bromide.

Separation of nicotine and nornicotine.[93] To a borosilicate column 20 mm. × 75 cm., constricted at one end to hold a perforated aluminum disc covered by a small wad of cotton, add starch equivalent to 5 grams of anhydrous material suspended in 35-40 ml. of water-saturated 1-butanol. Force out excess solvent by gentle air pressure until level with the top of the starch. Force a tight cotton plug down to the starch. Wash out excess butanol with about 25 ml. of hexane.

Pass the sample in hexane through the column. The nicotine goes through, while the nornicotine is retained. Then elute the nornicotine with chloroform.

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[93] Forrest G. Houston, *Ibid.* **24**, 1831-2 (1952).

Procedure—*By cyanogen bromide.* Prepare the cyanogen bromide reagent fresh for every analysis by titrating 25 ml. of cold saturated bromine water with 10 per cent sodium cyanide solution, avoiding excess. Bring to room temperature and, with a glass electrode, adjust to pH 7 with 0.1 per cent sodium cyanide solution and then to pH 4.1-4.2 with 1:180 sulfuric acid.

Dilute an aliquot containing not more than 0.08 mg. of nicotine and 0.15 mg. of nornicotine to 6 ml. with water. Add 2 ml. of 22 per cent potassium dihydrogen phosphate solution.

Heat at 80° for 5 minutes. Remove, add 2 ml. of cyanogen bromide reagent, and return to the 80° bath in exactly 15 seconds. Leave in the bath for 2 minutes and 45 seconds; then chill in ice for 1 minute. Read nicotine against a sample blank at 375 mμ within 4 minutes after removal from the cooling bath. Apply a correction obtained by reading nornicotine at 375 mμ. Read nornicotine in the solution at 540 mμ. Correction for nicotine is not necessary.

By aniline and cyanogen bromide. To 8 ml. of sample solution containing not over 3 mg. of nicotine and nornicotine, add 2 ml. of 1 per cent aqueous aniline solution in 0.2 *N* phosphate buffer for pH 6.1 (Vol. I, page 176). Add 1 ml. of 10 per cent cyanogen bromide solution. Color develops rapidly. Read after 5 minutes at 470 mμ against a reagent blank.

By β-naphthylamine and cyanogen bromide. To 3 ml. of sample containing 0.001-0.01 mg. of nicotine and nornicotine, add 0.2 ml. of a 5 per cent solution of potassium acetate in ethanol. Follow with 0.5 ml. of 0.2 per cent β-naphthylamine in ethanol and 0.3 ml. of cyanogen bromide reagent, mixing after each addition. Read at 480 mμ 6-8 minutes later at 20-22° against a reagent blank and correct for a sample blank.

By benzidine and cyanogen bromide. The sample is in 3 per cent sodium acetate as buffer. To 5 ml. of this solution add 2 ml. of cyanogen bromide reagent. Cool in ice for 45 minutes. Add 4 ml. of 0.05 per cent benzidine in ethanol dropwise. The color reaches its maximum in 7-13 minutes and remains for about 1 minute. Hence read at 420 mμ against a reagent blank at 1-minute intervals, starting 7 minutes after the addition of reagent.

By sodium nitroprusside and ammonium hydroxide. To 2-3 ml. of very dilute nicotine solution, add 0.1-0.2 gram of potassium persulfate and 1 drop of 1 per cent silver nitrate solution. Boil on a water bath

for 1 minute, shake, and cool to 10-20°. Add 0.05-0.15 gram of sodium thiosulfate, shake until dissolved, and follow with 2-4 drops of 10 per cent sodium nitroprusside solution. Make alkaline with 1:1 ammonium hydroxide. Read at 494 mμ in 2-3 minutes against the yellow reagent blank.

By silicomolybdic acid. Dilute the sample solution with 2:7 hydrochloric acid to a concentration of 1:35 hydrochloric acid. Add 2 ml. of silicomolybdic acid reagent (Vol. III, page 368) and let stand for at least 2 hours. Filter on a paper disc in a Gooch crucible. Wash the precipitate with 1 ml. of a wash liquid containing 10 per cent of sodium chloride in 1:70 hydrochloric acid. Repeat the washing with 0.5 ml. and 0.5 ml. of the same wash liquid, stirring up the precipitate each time. Discard the filtrate and washings. As reducing solution, add 1 gram of glycine to 20 ml. of 20 per cent sodium sulfide solution and mix. Add 5 ml. of concentrated ammonium hydroxide in several portions with shaking. Dilute to 100 ml. and mix. Add 5 ml. of the reducing agent to the precipitate and stir. A blue solution is produced immediately. Filter the solution and wash with small amounts of water until the liquid comes through colorless. Dilute to 25 ml. or 50 ml. Keep at 45° for 30 minutes and read against a reagent blank.

By bromothymol blue. Add 0.5-2.5 ml. of alkaloid solution to a mixture of 10 ml. of water, 2 ml. of 0.04 per cent bromothymol blue solution, and 5 ml. of saturated boric acid solution. Follow with 15 ml. of chloroform. Shake for 1 minute, separate the chloroform layer, and filter. Read with a band around 400-430 mμ.

Nephelometrically as phosphotungstate. To 10 ml. of neutral sample containing 0.1-1 mg. of nicotine and nornicotine add 0.2 ml. of 1:5 sulfuric acid and 0.2 ml. of 10 per cent phosphotungstic acid solution. Warm without boiling until clear and chill with gentle shaking in cold water to reestablish the turbidity. Read photometrically against data established under identical conditions. The threshold of opalescence is about 0.0005 mg. per ml.

2-(3-Pyridyl)pyrrolidine, Nornicotine

Nornicotine is usually included with nicotine in determinations. A method for its separate determination by cyanogen bromide is described under nicotine. The violet color of nornicotine with 1,3-diketohydrindene hydrate, ketone, and tannic acid is given by either the synthetic or

natural form.[94] Nicotine and anabasine do not interfere significantly. They can be chromatographed satisfactorily by starch, a technic described under nicotine (page 465).

Procedure—Dilute a sample in acetone containing 0.1-0.5 mg. of nornicotine to 5 ml. with acetone. Add 15 ml. of diisopropyl ketone, 2 ml. of 2 per cent *p*-hydroxybenzoic acid in diisopropyl ketone and 2 ml. of 0.3 per cent 1,3-diketohydrindene hydrate in diisopropyl ketone. Mix, store in the dark for 1 hour, and read at 540 mμ.

6,7-Dimethoxy-1-veratrylisoquinoline, Papavarine

Papavarine is read fluorometrically in the absence of other alkaloids.[95]

Procedure—Warm 4 ml. of sample containing 0.04-0.4 mg. of papavarine to 80° for 1 hour. Add 0.2 ml. of 1:9 sulfuric-acetic acid mixture and maintain at 80° for 2 hours. Read fluorometrically.

Physiostigmine, Eserine

Physiostigmine is unstable when heated in alkaline solution. After decomposition to eseroline and rubreserin, it forms a yellow dye which is suitable for reading at a level of 0.002-0.1 mg. per ml.[96] The same test substance on hydrolysis with sodium hydroxide liberates methylamine. Methods of estimation of aliphatic amines are then applicable to the steam distillate.[97] Still another method [98] involves alkaline oxidation by ferricyanide.

Samples—*Aqueous solutions.* Dilute with water to 0.01-0.1 mg. per ml. Thus ampoules containing 1 mg. per ml. are conveniently diluted 1:25 for alkaline oxidation.

Oil solutions. Dilute the sample with chloroform to below 0.1 mg. of alkaloid per ml. Shake 10 ml. of this solution 5 times with 5-ml. portions of 1:20 hydrochloric acid. Decant the acidic phase through a filter for use as sample for alkaline oxidation.

Blood. Hemolyze 10 ml. of citrated blood with 10 ml. of water. Precipitate proteins with 10 ml. of 20 per cent trichloroacetic acid solu-

[94] Louis Feinstein and Edward T. McCabe, *Ibid.* 23, 924-5 (1951).
[95] Erich Wegner, *Pharmazie* 5, 445-7 (1950).
[96] Friedrich Meyer, *Ibid.* 5, 111-13 (1950).
[97] Hans Hellberg, *Svensk Farm. Tid.* 51, 560-9 (1947).
[98] Inge Ehrlén, *Farm. Revy* 47, 519-24 (1948).

tion. Filter and wash the precipitate with 5 ml. of 7 per cent trichloroacetic acid solution. Make the filtrate alkaline with 30 per cent potassium hydroxide solution and extract with 30, 30, and 30 ml. of chloroform. Centrifuge to break emulsions. This extraction must be carried out promptly and must extract the physiostigmine or decomposition products before the yellow dye stage is reached. The latter is insoluble in chloroform. Extract the combined chloroform extracts with 5, 5, and 5 ml. of 1:20 hydrochloric acid. Use the combined acid extracts or an aliquot as sample for alkaline oxidation.

Plasma or serum. Dilute 10 ml. with an equal volume of water and proceed as for blood from "Precipitate proteins with 10 ml. of 20 per cent trichloroacetic acid solution."

Urine. Clarify with lead acetate and remove excess lead with hydrogen sulfide. Then proceed as for blood from "Make the filtrate alkaline with 30 per cent . . . .."

Tissue. Triturate 10 grams of finely cut tissue with sea sand in a mortar and add 20 ml. of 20 per cent trichloroacetic acid solution. Complete as for blood from "Filter and wash the precipitate . . . ."

Procedure—*By alkaline oxidation.* Make 5 ml. of the unknown aqueous solution alkaline with 1 or 2 pellets of potassium hydroxide. The solution becomes red in a short time by heating immediately. Continue heating on a water bath with shaking. By heating, the color changes to yellow. After cooling, read the yellow color at 470 mμ against a reagent blank.

By ninhydrin. Mix an extract containing 0.01-0.1 mg. of physiostigmine with 2 ml. of 8 per cent sodium hydroxide solution and steam-distil the methylamine formed into 2 ml. of 1 per cent monobasic potassium phosphate. Add 1 ml. each of 0.4 per cent sodium hydroxide solution and freshly prepared 1 per cent aqueous ninhydrin reagent containing ascorbic acid. Heat in a boiling water bath and read the violet color at 570 mμ.

By ferricyanide. Mix a solution containing 1-4 mg. of physiostigmine with 1 ml. of 2 per cent potassium ferricyanide solution and 1 ml. of 4 per cent sodium hydroxide solution. Dilute to 50 ml. with water and after 10-15 minutes read at 480 mμ against a reagent blank.

Pilocarpine

Pilocarpine is separated from quinine and other alkaloids by saponifying its lactone group with alkali to form a water-soluble salt. The other

alkaloids are extracted by chloroform, leaving the pilocarpine.[99] On addition of acid this is developed with bichromate and hydrogen peroxide.[100] Pilocarpine is precipitated by silicotungstic acid, as are most alkaloids. In the absence of other alkaloids it is determined by so precipitating and determining the excess by reduction with titanous chloride (page 428). Another method is as the reinickate (page 428).

Sample—*Pilocarpine or a salt.* Dissolve in water to give a concentration of about 2 mg. per ml. To 10 ml. of aqueous solution of alkaloids, add 0.1 gram of sodium bisulfite and 3 ml. excess of 10 per cent sodium hydroxide solution. Mix, let stand for 5 minutes, and extract with 5 successive 20-ml. portions of chloroform. Wash the combined extracts with 5 ml. of water and add the wash water to the residual alkaline solution. Discard the chloroform extracts.

Add 2 ml. excess of concentrated hydrochloric acid and let the aqueous solution stand for 15 minutes. Add a slight excess of 1:1 ammonium hydroxide and extract immediately with 5 successive 20-ml. portions of chloroform. Filter the extracts through a plug of cotton and evaporate the chloroform on a steam bath. Dissolve the residue in water and dilute to 25 ml. Determine the approximate amount of pilocarpine by titrating a 10-ml. aliquot to methyl red with 0.02 N acid of which each ml. is equivalent to 4.16 mg. of pilocarpine.

Procedure—*By bichromate.* Dilute an aliquot containing 0.2-2 mg. of pilocarpine to 10 ml. and add 1 ml. of acetic acid and exactly 20 ml. of chloroform. Then add 1 ml. of 5 per cent potassium bichromate solution and 1 ml. of 3 per cent hydrogen peroxide and shake vigorously for about 30 seconds. Allow the layers to separate and filter the chloroform layer, protecting from direct sunlight and daylight as much as possible. Read at 560 mμ against chloroform.

Quinidine

Determine as the reinickate by a technic described under alkaloids in general (page 428).

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[99] Irwin S. Shupe, *J. Assoc. Official Agr. Chem.* **24**, 757-66 (1941).

[100] John W. Webb, Ray S. Kelley, and Arthur J. McBay, *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 278-9 (1952).

Quinine

If other alkaloids which give a precipitate with picric acid are absent, quinine can be precipitated with a large excess of this acid. The picrate is somewhat soluble in water but, by appropriate manipulation, error from this source is minimized. The quinine picrate is then dissolved in ammonium hydroxide for direct comparison.[101] The quinine present with ammonium picrate in the solutions to be compared is removed by extraction with chloroform. An indirect method is by comparison of the residual picric acid present in the solutions after precipitation. Both methods are accurate to 2 per cent.

Quinine is precipitated by phosphotungstic acid and the amount of quinine estimated by reduction with titanous chloride to a tungsten blue. The method is similar to that with silicotungstic acid. Other substances precipitated by phosphotungstic acid such as strychnine, emetine, and cinchonine must be absent. The method is accurate within 1 per cent. For the corresponding procedure with silicotungstic acid, follow either method of preparation of strychnine samples and the procedure for application of this reagent to strychnine (page 481). The method is accurate to 2 per cent.

An aqueous solution of quinine is oxidized in neutral or slightly acid solution by bromine water or chlorine water. On adding ferricyanide solution and rendering alkaline with ammonium hydroxide the erythroquinine formed by oxidation is extracted with chloroform and estimated by its rose to red color.[102] The color is stable for 2-3 hours. Phenylhydrazine hydrochloride can replace the potassium ferricyanide. The method gives similar results with cinchonine and thalleioquinine. Brucine gives a rose color but can be distinguished by a difference in the quality of the initial color. Narceine gives a yellow color extracted with chloroform. Strychnine, morphine, narcotine, papaverine, cocaine, atropine, hydrastine, aspirin, urethane, veronal, caffeine, acetanilide, exalgine, and phenacetine do not give the reaction. If quinine is associated with substantial amounts of antipyrine or pyramidon, yellow rather than red is developed. Very small amounts do not interfere. The method will detect 1 ppm. and, if more than 100 ppm. are present, it must be modified

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[101] C. A. Rojahn and Rudolf Seifert, *Arch. Pharm.* **268**, 499-520 (1930).

[102] R. Monnet, *J. pharm. chim.* **18**, 59-65 (1933); George W. Hargreaves, *J. Am. Pharm. Assoc.* **15**, 100-5 (1926); R. Monnet, *J. pharm. chim.* **18**, 94-6 (1933); R. Moers, *J. pharm. Belg.* **4**, 219-27 (1949).

by use of more chloroform because the erythroquinine will not dissolve completely.

Quinine gives a yellow color with *p*-nitrodiazobenzene reagent [103] or a red color soluble in chloroform with 3 per cent eosin.[104] The color is also given by cinchonine, ephedrine, eserine, pilocarpine, and atropine.

The unstable colloidal brown color produced by reaction between quinine and potassium bismuthous iodide is used for estimation of the quinine.[105] Other alkaloids must be absent as provided for in the method of preparation of samples. The method will detect 0.2 mg. per liter of sample with accuracy within 4 per cent. The method as the reinickate is that already described (page 428).

Quinine is estimated nephelometrically by silicotungstic acid.[106] The reagent reacts with atrophine, morphine, strychnine, and probably with other alkaloids. The method will estimate 0.005-0.01 mg. of quinine per ml. with an average accuracy of 3 per cent. The sample is also read fluorometrically.[107] Quinidine gives the same fluorescence, but other cinchona alkaloids do not fluoresce appreciably. It is read in the ultraviolet at 250 mμ [108] or 347.5 mμ.[109] At the latter wave length interferences are at a minimum.

Iodine produces turbidity in saturated ammonium sulfate solutions of quinine, quinidine, or cinchonine, depending on the concentration of total alkaloid.[110] The sensitivity of the method varies with the concentration. From 0.001 to 0.045 mg. of alkaloid in 5 ml. can be determined. For quantities from 0.001 to 0.03 mg., a difference of 0.001 mg. appears quite sharply. From 0.03 to 0.045 mg. the same difference may be de-

[103] Alfredo Dellepiane, *Mon farm.* **51**, 161-3 (1945).

[104] R. O. Prudhomme, *Bull. soc. path. exotique* **31**, 929-32 (1938); *J. pharm. chim.* **1**, 8-17 (1940); C. Paul, L. Truffert, and Soro-Besnard, *Ann. méd. légale, criminol., police sci., méd. sociale, et toxicol.* **30**, 27-32 (1950).

[105] Edward B. Vedder and John M. Masen, *Am. J. Tropical Med.* **11**, 217-29 (1931); Henry Foy and Athena Kondi, *Ann. Trop. Med.* **29**, 497-515 (1935).

[106] Granvil C. Kyker, Bailey D. Webb, and James C. Andrews, *J. Biol. Chem.* **139**, 551-67 (1941).

[107] Bernard B. Brodie and Sidney Udenfriend, *J. Pharmacol.* **78**, 154-8 (1943); Carlos A. Gran, Juan A. Migo, and Ricardo Elicabe, *Rev. farm.* (Buenos Aires) **91**, 193-200 (1949); Archie L. Edgar and Maurice Sokolow, *J. Lab. Clin. Med.* **36**, 478-84 (1950); Fernand Chateau, *Ann. fals. et fraudes* **44**, 101-3 (1951).

[108] Edward S. Josephson, Sidney Udenfriend, and Bernard B. Brodie, *J. Biol. Chem.* **168**, 341-4 (1947).

[109] J. Carol, *J. Assoc. Official Agr. Chem.* **26**, 238-41 (1943).

[110] H. W. Acton and H. King, *Biochem. J.* **15**, 53-9 (1921); A. C. Roy, *Indian J. Medical Research* **14**, 129-33 (1926).

tected but less easily. It is even possible to see differences of 0.0005 mg. over a range of 0.005 to 0.02 mg. in 5 ml. Suspensions of the higher concentrations are compared immediately after mixing; those of 0.01 mg. or less in 5 ml., after 2 to 5 minutes. As little as 0.001 mg. can be detected in 2 ml. by examining the suspension vertically.

Quinine and quinidine both absorb at 318 and 348 mμ. Similarly cinchonine and cinchonidine absorb at 235 and 316 mμ. Therefore, they are determined in acid solution by simultaneous equations.[111] As given quinine and quinidine are referred to as quinine. The same coalescence applies to cinchonine.

Sample—*Urine.* Add 10 drops of 1:10 ammonium hydroxide to 20 ml. of urine to render it slightly alkaline. Extract the quinine with 2, 2, and 1 ml. of chloroform. Dry the chloroform extracts with anhydrous sodium sulfate and filter through cotton. Use the chloroform solution for oxidation by bromine.

For estimation by potassium bismuthous iodide, extract the quinine from the sample with ether and evaporate the extract. Add 5 ml. of 1:78 sulfuric acid saturated with zinc sulfate, to the quinine residue. Warm in a boiling brine bath for 3 minutes to dissolve the quinine and filter while hot. Cool and use as sample solution.

For nephelometric estimations with silicotungstic acid, assemble the apparatus shown in Figure 19. Make the urine sample up to pH 9-10. Add through the funnel and add ether slowly until about 15 ml. overflows into the extractor jacket. Reflex for about 4 hours at such a rate that 25-35 drops per minute drop from the bottom of the sample tube. Place the latter so that ether flows down its outer surface instead of down the wall of the extractor jacket. Remove the sample tube from the jacket and place the jacket in a water bath at 50-60°, at a depth of 5 cm. Keep in the bath for 15 minutes to evaporate all ether and then transfer to a 100° bath for 5 minutes.

Cool and add a volume of 1:300 hydrochloric acid equal to the volume of sample which was extracted. Return the jacket to a 55° bath for 10 minutes, mix gently, and place in a 100° bath for 5 minutes. This type of heating causes complete solution of quinine and negligible evaporation of the solvent. Stopper, cool to room temperature, and rotate the jacket so that the condensate on the walls is mixed with the contents

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[111] Helen S. Grant and J. H. Jones, *Anal. Chem.* 22, 679-81 (1950).

of the tube. Remove any turbidity by filtering through glass wool at a rate of 1 ml. per minute.

Blood, serum, plasma, corpuscles. Follow the technic for urine for development with silicotungstic acid but do not make alkaline.

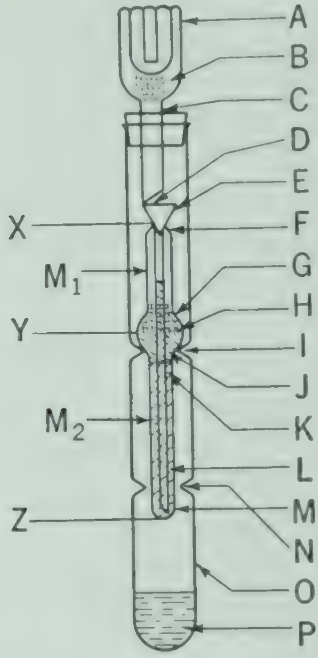

FIG. 19. Extraction apparatus

The parts and assembly of the extractor unit as employed for the extraction of blood or urine are described as follows: **A.** Hopkins condenser. **B.** and H. Tufts of fiber glass. **C.** Hole in cork stopper bored slightly off center. **D.** Tip of condenser touching side of funnel. **E.** 20 mm. funnel with a stem 180 mm. in length and 4 mm. outside diameter (used interchangeably with sample tubes constructed for 2, 5, and 10 ml. samples). **F.** Punch marks for the support of the funnel, **G.** Overflow for ether, 4 mm. in diameter. **I** and **N.** Punch marks 190 mm. and 105 mm. respectively from the bottom of the extractor jacket for support of the sample tube. **J.** Ether layer. **K.** Ether-blood emulsified zone. **L.** Sample of blood or urine. **M.** Sample tube (dimensions: from **X** to **Y,** 70 mm.; from **Y** to **Z,** 110 mm.; outside diameter 16 mm. at M_1, 22mm. at **Y,** and 9, 12, and 16 mm. respectively at M_2 for 25, and 10 ml. sample tubes). **O.** Extractor jacket 320 mm. in length and 32 to 35 mm. outside diameter. **P.** Ether reservoir, 15 to 20 ml. The scale drawing of the apparatus shows a sample tube for a 5 ml. sample; in this and the other sample tubes, the proper sample volume stands in a column 80 to 85 mm. high when the funnel stem filled with ether is in place.

For fluorescence, dilute 1 ml. of plasma with 39 ml. of water and add 10 ml. of 20 per cent metaphosphoric acid solution with shaking. After 15 minutes, centrifuge.

For reading in the ultraviolet, mix 1-4 ml. of plasma with 1 ml. of 10 per cent sodium hydroxide solution. Add 15 ml. of ethylene dichloride

and shake mechanically for 10 minutes. Centrifuge and separate the aqueous phase to waste. Shake the ethylene dichloride layer for 5 minutes with 45 ml. of 0.8 per cent borax solution. Discard the washings and repeat. Dilute the solvent phase to a known volume for aliquoting.

Feces. Weigh the entire sample and transfer to a large vessel which can be tightly closed. Add 5-7 ml. of concentrated ammonium hydroxide and work into a thin paste with distilled water. All lumps must be broken up. Add ether to form a 5-6 cm. layer over the paste. Shake mechanically for 10 minutes and let stand for one hour with occasonal shaking. Centrifuge and remove the ether layer. Add more ether and extract again. Repeat a third time. Filter the combined ether extracts and evaporate to dryness. Take up the residue with 5 ml. of 1:10 hydrochloric acid. Heat on a water bath and filter clear while hot. If the filtrate is red-brown, too little ammonia has been used. In that case shake with a little Permutit and filter. Develop the sample nephelometrically with silicotungstic acid.

Bark. Mix 0.1 gram of finely ground bark with 0.2 gram of calcium oxide and 2 ml. of water. Dry at 100°, grind, and extract three times with warm chloroform. Filter and evaporate the chloroform to dryness. Take up in 3 ml. of 1:25 sulfuric acid. Filter and wash. Dilute to 10 ml. as sample. Develop by *p*-nitrodiazobenzene or by oxidation.

For reading fluorescently, mix 0.4 gram of bark mixed with 0.12 gram of hydrated lime and 3 ml. of 4 per cent sodium hydroxide. Extract with several successive portions of benzene. Place this over 10 ml. of 1:25 sulfuric acid and evaporate the benzene. Dilute the solution of quinine sulfate to 100 ml. for reading.

For quinine and cinchonine. Mix approximately 1 gram of powdered bark, a few ml. of 10 per cent sodium hydroxide solution, and 100 ml. of benzene, and weigh. Reflux gently for 3-6 hours. Cool and replace any weight of benzene that was lost. Separate that layer. Extract a 50-ml. aliquot of benzene solution with several small portions of 1:120 hydrochloric acid. Boil the combined acid extracts for 2-3 minutes to expel all of the benzene, cool, and dilute to 1 liter for reading in the ultraviolet. This solution should contain 10-60 mg. of the alkaloids per ml. The value probably includes related alkaloids.

Procedure—*By picric acid. Direct.* To 5 ml. of sample containing from 1 to 5 mg. of quinine per ml., add 0.8 ml. of 10 per cent sodium chloride solution to facilitate precipitation of quinine picrate. Add 6 ml. of 1 per cent picric acid solution. Mix well, let stand for 3 hours, and

centrifuge. Decant the upper layer and disperse the precipitate in 5 of distilled water to wash. Centrifuge and repeat the washing. To solid picrate add 15 ml. of 1:6.5 ammonium hydroxide and trans to a 50-ml. tube. Wash in water. Dilute to 50 ml. and add 5 ml. chloroform. Shake well and let stand until the chloroform separat usually about 2 hours. Read the aqueous layer against a water blank.

Indirect. To 5 ml. of solution containing 1 to 5 mg. of quinine ml. add 25 ml. of 20 per cent sodium sulfate solution and 5 ml. of 1 cent picric acid solution. Dilute to 50 ml. Let stand in a water ba at room temperature for 24 hours. Filter and read the filtrate again a blank to which quinine was not added. The estimation gives picric acid which has been removed by the quinine. This figure mul plied by 1.65 gives the value in terms of hydrated quinine or by 1. in terms of quinine sulfate.

By phosphotungstic acid. To 5 ml. of sample containing 1 to 5 m of quinine per ml., add 1 ml. of 10 per cent sodium chloride soluti and 1 ml. of 1:5 hydrochloric acid. Mix well and add 3 ml. of 10 p cent phosphotungstic acid solution. Centrifuge after 10 minutes ar decant. Disperse the precipitate in 4 ml. of water, 0.5 ml. of 10 p cent sodium chloride solution, and 0.5 ml. of 1:50 hydrochloric aci Centrifuge, decant, and wash again. Decompose the precipitates wit 2 ml. of 8 per cent sodium hydroxide solution. Dilute with water, filte wash the filter well, and dilute to 50 ml.

Neutralize 5 ml. of the prepared sample with 1:5 hydrochloric aci If a precipitate is formed, dissolve with a drop of 8 per cent sodiu hydroxide solution. Dilute to 20 ml. and add 1 ml. of 10 per cent gu arabic solution. Add 5 drops of 1:5 hydrochloric acid. Dilute 2 ml. o 10-15 per cent titanous chloride solution to 50 ml. with boiled wate Add 1 ml. of this reducing agent, dilute to 25 ml., and read.

By bromine water. To 5 ml. of filtered test solution add 0.5 ml. o saturated bromine water. Shake for 10 seconds, add 10 drops of con centrated ammonium hydroxide, and shake for 15 seconds. Dilute t 10 ml. with methanol. Read at 440 mμ against a reagent blank.

By potassium bismuthous iodide. As one reagent, shake 2 grams o gum arabic with 100 ml. of water until dispersion is complete. Heat i boiling brine for one hour to destroy reducing enzymes and filter while hot. Store in a well-stoppered bottle in a refrigerator. As potassium bismuthous iodide, dissolve 0.47 gram of bismuth oxide in 8 ml. of con-centrated hydrochloric acid. Dilute to 30 ml. with water. Dissolve 20

grams of potassium iodide in water and dilute to 70 ml. Mix the solutions as reagent. Incubate 3 ml. of sample at 20-25° for 5 minutes. Add 0.06 ml. of the prepared reagent and mix. Read within 2 minutes against a reagent blank.

By p-nitrodiazobenzene. The sample as submitted is dissolved in dilute sulfuric acid. Evaporate a 1-ml. aliquot by heating at 120° and then keep for 5 minutes at 180°. Cool, add 2 ml. of water and 1 ml. of *p*-nitrodiazobenzene reagent. Shake, add 10 drops of 30 per cent sodium hydroxide solution, and shake again. Add 10 drops of 1:3 sulfuric acid and 2 ml. of ethanol. Read against a reagent blank.

By eosin. Add 2 per cent eosin solution to the sample, buffer to pH 7 (Vol. I, page 176), and extract the red color with chloroform. Read against chloroform.

Nephelometrically by silicotungstic acid. Adjust 10 ml. of the sample solution to a reading of 100, using a 400-mμ filter. Add 0.2 ml. of 10 per cent silicotungstic acid solution to the sample and heat in boiling water for 5 minutes. Cool for 5 minutes. Allow to stand at room temperature for 5 minutes and read.

In the ultraviolet. The sample will normally be an extract in organic solvent. Shake a 10-ml. aliquot of the solvent phase with 4 ml. of 1:360 sulfuric acid for 3 minutes and centrifuge. Read the acid layer at 250 mμ against a reagent blank.

Fluorometrically. Make an aqueous sample alkaline with 1:1 ammonium hydroxide. Evaporate if necessary and extract with chloroform. Wash the chloroform extract with water and re-extract with 1:10 sulfuric acid. Read fluorometrically. Alternatively compare an acid sample against 50 mg. of quinine per liter.

Reading quinine and cinchonine in the ultraviolet. Read the acid solution at 316 and 348 mμ. From this and the density of standard quinine and cinchonine solutions calculate the alkaloid content as follows:

$$D_{316} = XD_q^{316} + YD_c^{316}$$
$$D_{348} = XD_q^{348} + YD_c^{348}$$

where

X = quinine-type alkaloids
Y = cinchonidine-type alkaloids
D_{316} and D_{348} = optical densities of the samples at 316 mμ and 348 mμ

D_q^{316}, D_q^{348}, D_c^{316} and D_c^{348} = optical densities per unit weight of the two standards at the respective wave lengths

6-Hydroxy-7-methoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline, Salsoline

Color development by sodium nitrite and ammonium hydroxide is applicable to salsoline in the absence of morphine.[112]

Procedure—To 10 ml. of aqueous sample add 12 drops of 1:4 hydrochloric acid and 1.5 ml. of 10 per cent sodium nitrite solution. After 5-6 minutes, add 3 ml. of 1:2 ammonium hydroxide and read at 532 mμ against a reagent blank.

Scopalamine, Hyoscine

For details of a method with bromocresol purple see atropine (page 430). In the absence of interfering alkaloids determine scopalamine by *p*-dimethylaminobenzaldehyde as also described for atropine (page 431). Alternatively determine as the reineckate, also described under alkaloids in general (page 428).

Senna Drugs

The active principles of senna drug are extracted and oxidized in alkaline solution with hydrogen peroxide.[113]

Procedure—Reflux 50-100 mg. of drug or extract with 1 ml. of concentrated hydrochloric acid and 7.5 ml. of glacial acetic acid for 15 minutes. Cool and add 30 ml. of ether. Shake and filter through a paper wet with ether. Extract the filtrate with 40 ml. of water. Further extract with 30, 10, and 10 ml. of 10 per cent sodium bicarbonate solution. Sennidins are left behind in the ether extracted. Combine the aqueous extracts, cover wth 40 ml. of ether, and acidify with 1:1 sulfuric acid. Separate the ether and extract the aqueous-acid layer with ether until the ether extracts total 100 ml. Extract a 10-ml. aliquot of ether extract with 10 ml. of 0.4 per cent sodium hydroxide solution. Add 0.2 ml. of 3 per cent hydrogen peroxide to the alkaline extract and heat in boiling water for 4 minutes. Cool and read against water.

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112 D. V. Bezuglyĭ, *Med. Prom.* (*USSR*) **1949**, No. 4, 33-5.

113 H. Auterhoff, *Arzneimittel-Forsch.* **1**, 412-14 (1951).

Sparteine

Determine as the reinickate by a method described under general (page 428).

Stramonium alkaloids

The mixed alkaloids are determined exactly the same as mixed belladonna alkaloids (page 432).

Strychnine

Strychnine, when reduced by zinc and hydrochloric acid, gives a red color when reacted with sodium nitrite.[114] Resorcinol and quinine interfere. The method will determine 0.01 mg.

Strychnine reacts with ammonium vanadate in sulfuric acid to give a color which, on dilution with water, is pink to red.[115] The color is permanent for several days but changes slowly to violet. Morphine, colchicine, yohimbine, ouabain, acetanilide, and cryogenin give a reddish or violet coloration which, on dilution, turn to pale yellowish which cannot be mistaken for the color given by strychnine. With 6 parts of quinine for 1 part of strychnine there is little effect on the intensity and stability of the coloration. From 6-30 parts of quinine there is a reduction of color up to 50 per cent. With 60 or more parts of quinine the reaction is negative. There are technics for separation from quinine.

Strychnine is precipitated quantitatively with silicotungstic acid. The solution of the precipitate so obtained, by reduction with titanous chloride, gives a blue color suitable for estimation, particularly when stabilized with a colloid.[116] Quinine, emetine, and cinchonine must be absent. Accuracy to ±1 per cent is obtainable. As another version, the solution from which the silicotungstate has been precipitated is reduced with titanous chloride. The method is applicable to many alkaloids in the absence of others.[117] Strychnine is also precipitated with phospho-

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114 Paul Malaquin, *J. pharm. chim.* (6) **30**, 546-9 (1910); Georges Deniges, *Bull. soc. chim.* **9**, 537-42 (1911); André Francois, *Bull. soc. pharm. Bordeaux* **68**, 158-63 (1930); M. Ya. Tropp and P. S. Kraïzman, *Trans. Ukrain. Inst. Exptl. Pharm.* **1**, 154-61 (1938).

115 E. Scandola, *Estr. boll. soc. med. chirurg. di Pavia* 1910; S. K. Rasmussen, *Dansk. Tids. Farm.* **16**, 11-19 (1942); Ch. Bedel, *Ann. pharm. franc.* **1**, 104-6 (1943); Marc Chambon, *Ibid.* **4**, 89-92 (1946).

116 A. Travers, *Compt. rend.* **166**, 416 (1918); C. A. Rojahn and Rudolf Seifert, *Arch. Pharm.* **268**, 499-520 (1930).

117 M. Mascre and J. Loiseau, *Bull. sci. pharmacol.* **48**, 273-80 (1941).

tungstic acid and estimated by reduction to a blue color in much the same way as with silicotungstic acid. Quinine, emetine, and cinchonine must be absent. The method is accurate to ±2 per cent.

In the absence of other alkaloids which give a precipitate, strychnine is precipitated with picric acid and the amount of strychnine determined from the color of the combined picric acid. Strychnine forms a precipitate with potassium ferrocyanide. By decomposition, the iron content is used as an indication of the strychnine content.[118] Other substances precipitated by ferrocyanide interfere. The method is accurate to ±2 per cent. Strychnine gives a color with ceric sulfate in sulfuric acid which may be estimated.[119] The method as the reinickate appears under alkaloids in general (page 428).

Strychnine and brucine in dilute sulfuric acid are read in the ultraviolet.[120] The maximum for strychnine is at 263.5 mμ; that for brucine at 302 mμ.[121] They are also determined by equations from readings at 252 mμ and 262 mμ.[122]

Samples—*Solutions.* Dilute the solution to about 0.04 mg. of strychnine per ml. Use 1 ml. as sample. Develop by reduction and nitrite. In the case of strychnine arsenate increase the period of reduction given under the procedure.

For development by ammonium vanadate, add to a sample containing about 2 mg. of strychnine, one-fifth the volume of 1:1 ammonium hydroxide. Extract with 4 ml. and 4 ml. of chloroform and use the combined extracts.

Powdered nux vomica.[123] Mix 0.1 gram with 1 ml. of concentrated hydrochloric acid and dilute to 100 ml. Shake and filter. Use 2 ml. as sample for development by reduction and nitrite.

Powdered St. Ignatius' Bean. Follow the same method as for powdered nux vomica.

Extract of nux vomica. Treat 0.1 gram of extract by the method for powdered nux vomica and use 0.5 ml. of the final solution as sample.

[118] C. A. Rojahn and Rudolf Seifert, *Arch. Pharm.* 268, 499-520 (1930).

[119] E. R. Cole, *J. Proc. Roy. Soc. N. S. Wales* 81, Pt. 4, 276-9 (1947).

[120] Paul Demoen and Paul Janssen, *J. pharm. Belg.* 7, 80-5 (1952).

[121] K. Jentzsch, *Scientia Pharm.* 19, 219-32 (1951).

[122] R. N. Bhattacharya and A. K. Ganguly, *J. Pharm. and Pharmacol.* 4, 485-90 (1952).

[123] Cf. John Allen and Noel L. Allport, *Quart. J. Pharm. Pharmacology* 13, 252-60 (1940).

Tincture of nux vomica. Dilute 1 gram of tincture to 100 ml. and use 2 ml. of this dilution as sample by reduction and nitrite.

Strychnine sulfate granules. Heat 1 granule in 10 ml. of water to boiling. Dilute to 100 ml. and use 4 ml. of this solution as sample by reduction and nitrite.

Much quinine present. Make the solution containing about 10 mg. of strychnine slightly acid and precipitate quinine by dropwise addition of saturated solution of sodium potassium tartarate. Filter and add 1:1 ammonium hydroxide to the filtrate until definitely alkaline. Extract with 20, 10, and 10 ml. of chloroform. Evaporate the chloroform extracts and dissolve the residue in 25 ml. of 1:10 sulfuric acid. Precipitate the strychnine by dropwise addition of excess of 4 per cent potassium ferrocyanide solution. Centrifuge and wash the precipitate. Take up the precipitate in 10 ml. of 1:10 ammonium hydroxide. Extract this with 5 ml. and 5 ml. of chloroform and develop with ammonium vanadate.

Procedure—*By reduction and nitrite.* Dilute a sample containing about 0.04 mg. of strychnine to 4 ml. Add 4 ml. of concentrated hydrochloric acid and 2 grams of zinc amalgam. Heat in boiling water for 10 minutes. Decant, cool, and add 1 drop of 0.1 per cent sodium nitrite solution. Read against a reagent blank.

By ammonium vanadate. Evaporate a sample containing approximately 2 mg. of strychnine to dryness in a porcelain evaporating dish. As reagent, dissolve 0.5 gram of ammonium vanadate in 100 ml. of 2:1 sulfuric acid. Add 2 ml. of this to the residue and stir well until the residue dissolves to give a red color. Dilute to 10 ml. with water and read against a reagent blank.

By silicotungstic acid. To 5 ml. of sample solution, containing 1 to 5 mg. of strychnine per ml., add 1 ml. of 10 per cent silicotungstic acid. After 3 hours with occasional stirring, centrifuge and decant. Prepare a wash liquid from 60 ml. of water, 10 ml. of 10 per cent sodium chloride solution, and 10 ml. of 1:5 hydrochloric acid. Disperse the precipitate in 3 ml. of this, centrifuge, and decant. Repeat this washing procedure. This removes excess silicotungstic acid. Add 2 ml. of 1:5 ammonium hydroxide and heat in a water bath for 10 minutes. Centrifuge to separate strychnine and wash the precipitate with several portions of water. Mix the filtrate with 5 ml. of chloroform to extract strychnine and dilute the aqueous phase to 50 ml.

As reducing reagent, boil 1 ml. of 10-15 per cent aqueous titanous chloride solution with 1 ml. of concentrated hydrochloric acid and dilute to 100 ml. with boiled water. This solution will keep only about 1 day. Mix 5 ml. of the aqueous sample and 1 ml. of 1:10 hydrochloric acid, and dilute to 20 ml. Add 1 ml. of titanous chloride reagent and dilute to 25 ml. Read against a reagent blank.

By phosphotungstic acid. Mix 5 ml. of sample containing 1 to 5 mg. of strychnine per ml. with 1 ml. of 10 per cent sodium chloride solution, followed by 1 ml. of 1:5 hydrochloric acid. Add 3 ml. of 10 per cent phosphotungstic acid solution. Mix and let stand for 10 minutes. Centrifuge and decant. Disperse the precipitate in 4 ml. of water, 0.5 ml. of 10 per cent sodium chloride solution, and 0.5 ml. of 1:5 hydrochloric acid. Centrifuge, decant, and wash again. Decompose the precipitate with 2 ml. of 8 per cent sodium hydroxide solution and add 5 ml. of chloroform to extract the strychnine. Dilute the aqueous layer to 50 ml.

Neutralize 20 ml. of sample solution with 1:5 hydrochloric acid and add 0.5 ml. in excess. Dilute to 25 ml. Dilute 2 ml. of 10-15 per cent titanous chloride solution to 50 ml. with boiled water. Add 5 ml. of this to the sample solution and read against a reagent blank.

By picric acid. To 5 ml. of sample containing 1-5 mg. of strychnine per ml., add 3 ml. of 1 per cent picric acid solution. Stir occasionally for 3 hours. Keep the tube stoppered to prevent evaporation. Centrifuge and decant. Wash the precipitate with 5 ml. of water. Dissolve the precipitate with 15 ml. of 1:5 ammonium hydroxide and dilute to 50 ml. Add 5 ml. of chloroform and shake. Read the aqueous ammonium picrate solutions at 460 mμ against a sample blank.

By ferrocyanide. Mix 5 ml. of strychnine solution containing 1 to 5 mg. of strychnine per ml. with 3 ml. of 1:18 sulfuric acid. Add 3 drops of fresh saturated sulfurous acid and 1 ml. of fresh, 10 per cent potassium ferrocyanide solution. Mix well and let stand for 1 hour. Centrifuge and decant. Mix 35 ml. of water, 15 ml. of 1:18 sulfuric acid, and 0.75 ml. of saturated sulfurous acid as a wash solution. Disperse the precipitate in 5 ml. of this solution. Centrifuge and repeat the washing twice. Disperse the precipitate in 5 ml. of concentrated sulfuric acid and heat with frequent addition of saturated bromine-water until a clear solution is obtained. Dilute to 100 ml. and develop the iron content (See Vol. II, pages 307-332).

Thebaine, Paramorphine

Thebaine is coupled in sulfuric acid solution with diazobenzene sulfonic acid.[124] Codeine and narcotine interfere.

Procedure—Make the sample solution 1:49 with sulfuric acid, heat for 28 minutes in a water bath, and cool. Couple with diazotized sulfanilic acid reagent (page 419) and read at once at 570 mμ against a reagent blank.

3,7-Dimethylxanthine, Theobromine

Theobromine or theophylline are extracted from aqueous solution with chloroform containing 5 per cent of isopropanol. Uric acid is not so extracted. The solvent solution will give up its alkaloids on shaking with 0.4 per cent sodium hydroxide solution. Theophylline can then be read at 274 mμ or theobromine at 277 mμ. Each will interfere with the other.[125]

If caffeine is present, eliminate it by heating the solution at 100° for 30 minutes before reading. Barbiturates may interfere. The oxidation of caffeine by bromine gives a characteristic color also applicable to theobromine or theophylline.

Procedure—Extract the sample with chloroform containing 5 per cent of isopropanol. Shake the separated nonaqueous phase with a small volume of 0.4 per cent sodium hydroxide solution. Read theobromine at 277 mμ or theophylline at 274 mμ.

1,3-Dimethylxanthine, Theophylline

See the method for theobromine.

Trigonelline, Nicotinic acid N-methylbetaine

Trigonelline shows a maximum absorption in aqueous solution at 264.5 mμ which is proportional to concentration over pH 4 to 8.[126] Caffeine, chlorogenic acid, proteins, etc., absorbing in this region must be removed. No chemical precipitating agent can be used but sorbants

124 Erich Wegner, *Pharmazie* **5**, 33-5 (1950).

125 Jerome A. Schack and Samuel H. Waxler, *J. Pharmacol. Exptl. Therap.* **97**, 283-91 (1949).

126 R. G. Moores and Dorothy M. Greninger, *Anal. Chem.* **23**, 327-31 (1951).

remove the trigonelline for subsequent elution. Thus acid-washed Superfiltrol will remove all trigonelline and caffeine, permitting much of the chlorogenic acid to pass through. The balance is washed out with acid ethanol, leaving the trigonelline. Elution with ammonia of proper concentration leaves the caffeine on the sorbant. Potassium permanganate oxidation does not affect trigonelline. Zinc ferrocyanide precipitates trigonelline quantitatively, thus permitting determination of background absorption.

When trigonelline is hydrolyzed with alkali in alcoholic solution, methylamine splits off, yielding a dialdehyde. This condenses with various amines to give a deep-yellow as a measure of trigonelline.[127] The following amines are appropriate as reagents: *p*-aminoacetanilide, *p*-aminoacetophenone, 1-aminobenzimidazole, *p*-aminobenzoic acid, *p*-aminobenzophenone, 1-amino-2-naphthol, 2-amino-4-nitrophenol, *α*-aminopyridine, benzidine, cresidene, dianisidine, sulfanilic acid, tolidine, and *p*-toludine. Dianisidine is the most sensitive. Dextrose, in the presence of urea, interferes.

Another reaction is alkaline hydrolysis to glutaconaldehyde which is developed with benzidine or dianisidine.[128] For this follow the procedure for N-methylniacinamide (page 269). If that compound is also present, determine it separately fluorometrically and subtract from the total by benzidine.

Another reaction is by alkaline hydrolysis in the presence of a source of ammonia and subsequent development with cyanogen bromide and amines.[129] This assumes that nicotinic acid is formed. Conversions of 70 per cent are obtainable.

Sample—*Coffee.* Mix 2 grams of flaked green or roasted coffee in which all cells are ruptured, with 4 grams of Celite 545. Tranfer to a 6×80 mm. extraction tube having a glass fiber plug and a bed of the Celite. Percolate under vacuum at 7 ml. per minute with 200 ml. of 50 per cent ethanol. Dilute the extract to 200 ml.

[127] E. Kodicek and Y. L. Wang, *Nature* **148**, 23-4 (1941); J. C. Roggen, *Rec. trav. chim.* **61**, 209-11 (1942); *Chem. Zentr.* **1942**, **II**, 1610; Sidney W. Fox, Edna W. McNeil and Henry Field, Jr., *J. Biol. Chem.* **147**, 645-50 (1943).

[128] W. König, *J. prakt. Chem.* **69**, 105 (1904); E. Kodicek and Y. L. Wang, *Nature* **148**, 23-4 (1941); Sidney W. Fox, Edna W. McNeil, and Henry Field, Jr., *J. Biol. Chem.* **147**, 645-50 (1943).

[129] William A. Perlzweig, Edward D. Levy, and Herbert P. Sarett, *Ibid.* **136**, 729-45 (1940); Herbert P. Sarett, William A. Perlzweig, and Edward D. Levy, *Ibid.* **135**, 483-5 (1940).

For sorption place on the glass fiber plug of a 6 × 80 mm. column 10 mm. of Celite 545 followed by 6 grams of a mixture of 2 parts of the Celite with 1 part of Super Filtrol. Compress by tapping and applying vacuum. Percolate with 75 ml. of 1:18 sulfuric acid.

Fit a separatory funnel carrying 100 ml. of the coffee extract to the top of the column. Pass it through at about 5 ml. per minute. Follow it successively with 50 ml. of 50 per cent ethanol, 50 ml. of 2 per cent sulfuric acid in ethanol, and 50 ml. of ethanol, retaining liquid on top of the column at all times. Discard the trigonelline-free extract and washings.

Elute the column with 150 ml. of 3:97 ammonium hydroxide over about 20 minutes and dilute the eluate to 200 ml. with water for reading in the ultraviolet.

Procedure—*By dianisidine. Urine.* Add 2.5 ml. of water to 2.5 ml. of urine and follow with 15 ml. of methanol and 5 ml. of 40 per cent sodium hydroxide solution. Reflux for 30 minutes under a condenser attached to a soda lime tube and cool. Add 5 ml. of water. Adjust to pH 8 with concentrated hydrochloric acid and dilute to 50 ml. with methanol. Prepare a reagent by dissolving 2 grams of dianisidine in 100 ml. of acetone and 300 ml. of 1:20 hydrochloric acid. Mix a 9-ml. aliquot of sample with 1 ml. of reagent and read at 15-minute intervals at 520 mμ until a maximum absorption is reached.

Ultraviolet absorption. Green coffee. Dilute 50 ml. of eluate to 100 ml. and read at 264.5 and 325 mμ. Subtract the value at 325 mμ from the other and calculate from $E^{1\%}_{1\,\text{cm.}} = 297$.

Roasted coffee. Mix 50 ml. of eluate with 10 ml. of 1 per cent aqueous potassium permanganate. After 10 minutes add 3 ml. of 5 per cent aqueous sodium sulfite and mix. Add 1.5 ml. of glacial acetic acid and titrate with the sulfite solution until the manganese dioxide disappears. Dilute to 100 ml., filter discarding the first 10 ml. which is depleted by sorption, and read at 264.5 mμ.

To another 50-ml. portion of eluate add about 25 ml. of water and 1 ml. of glacial acetic acid. Add 5 ml. of a solution containing 21.9 per cent of zinc acetate dihydrate and 3 per cent of glacial acetic acid. With swirling add 5 ml. of a 10.6 per cent solution of potassium ferrocyanide trihydrate. Dilute to 100 ml. and shake. After about 5 minutes filter through a folded filter, discard the first 10 ml., and read at 264.5

mμ. Subtract this reading of trigonelline-free solution from the previous reading and calculate from $E^{1\%}_{1\,cm.} = 297$.

Turbocurarine Chloride

This alkaloid is read in the ultraviolet in aqueous solution at 280.5 mμ. Alternatively the reineckate is read in ethanol at 525 mμ[130] or color is developed with Millon's reagent.[131]

Procedure—*Reading in the ultraviolet.* Read a solution in ethanol at 280.5 mμ and calculate the concentration from $E^{1\%}_{1\,cm} = 101.6$.

As the reineckate. To a solution expected to contain 4-21 mg. of *d*-tubocurarine chloride pentahydrate per 10 ml. of water, add 1 ml. of 1:4 sulfuric acid. Follow with 3 ml. of 2 per cent reineckate salt solution, stopper with a rubber cap, and mix by inversion. Allow to stand for 1 hour to complete precipitation of the pink reineckate and centrifuge for 10 minutes. Decant the supernatant liquid and draw off excess liquid by pressing the mouth of the tube on filter paper. Wash the precipitate twice with 1-ml. portions of water, centrifuging each time. Decant and remove excess liquid as previously described. Dissolve the reineckate in absolute ethanol and bring the volume to 10 ml. If the resulting solution is turbid, clear by centrifuging, and read at 525 mμ.

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[130] Council in Pharmacy and Chemistry, *J. Am. Med. Assoc.* **138**, 821 (1948); Daniel Klein and Samuel M. Gordon, *J. Am. Pharm. Assn.* **38**, 438-41 (1949).

[131] A. M. Pryde and F. R. Smith, *J. Pharm. Pharmacol.* **1**, 192 (1949).

CHAPTER 12

ENZYMES[1]

Since there is no structural classification of enzymes, they have been listed alphabetically by their technical names. By definition the number of possible enzymes is so vast that those listed here are a mere start. There are, of course, no class reactions.

ALDOLASE, ZYMOHEXASE

Aldolase is the enzyme which catalyzes the conversion of 1 molecule of fructose diphosphate to 2 molecules of triose phosphate.[2] The triose phosphate formed is converted through methyl glyoxal to acetaldehyde by hot concentrated sulfuric acid, and the aldehyde is measured with *p*-hydroxydiphenyl. Anything which might liberate inorganic phosphate from the substrate, such as phosphatase, will cause interference. Color of the substrate is corrected by a blank.

The method is applicable to liver, kidney, spleen, muscle, plasma, tumor tissue, and isolated cell nuclei from liver, kidney, and pancreas, but is not accurate for crude tissue extracts or homogenates.

A modified procedure[3] provides for incubating the buffered test sample with fructose-1,6-diphosphate and hydrazine to fix the triose phosphates formed. Either reaction is stopped with trichloroacetic acid. An aliquot of the filtrate is then treated with 2,4-dinitrophenylhydrazine. Upon making the mixture alkaline again, the characteristic color is directly proportional to the enzyme concentration.

Procedure—*By p-hydroxydiphenyl.* Neutralize 0.02 per cent iodoacetic acid to pH 7 with 1 per cent sodium hydroxide solution. This blocks the action of 3-phosphoglyceraldehyde. Add 0.1 ml. of this to 0.7 ml. of 3.4 per cent disodium fructose diphosphate. Follow with 0.2 ml. of diluted enzyme solution, or homogenized tissue suspension, plasma, or isolated tissue cell nuclei and start timing the reaction. Incu-

[1] See Volume III, Chapter 1, for details of organization, condensation, etc.

[2] Alexander L. Dounce and G. Thannhauser Beyer, *J. Biol. Chem.* 173, 159-73 (1948).

[3] John A. Sibley and Albert L. Lehninger, *Ibid.* 177, 859-72 (1949).

bate the reaction mixture at 25° for 15 minutes. Stop the reaction by the addition of 4 ml. of 8 per cent trichloroacetic acid solution and centrifuge to separate the precipitated protein. Place 1 ml. of the supernatant liquid in ice-water and add slowly, with stirring, 6 ml. of concentrated sulfuric acid. Place in boiling water for 3 minutes and then cool to room temperature under tap water. Add 1 drop of 4 per cent cupric sulfate solution and 2 drops of 1.5 per cent *p*-hydroxydiphenyl in 0.5 per cent sodium hydroxide solution. Incubate at 27-30° for 30 minutes and then place in boiling water for 60-90 seconds. Cool under tap water, read at 560 mμ, and subtract a reagent blank.

By dinitrophenylhydrazine. Mix the sample at 38° with 1 ml. of 0.63 per cent aqueous tris(hydroxymethyl)aminomethane adjusted to pH 8.6 with 1:10 hydrochloric acid. Add 0.25 ml. of 1.7 per cent fructose-1,6-diphosphate solution, 0.25 ml. of 0.73 per cent hydrazine sulfate solution brought to pH 8.6 with 3 per cent sodium hydroxide solution, and sufficient water to make a total volume of 2.5 ml. After 30 minutes stop the reaction by the addition of 2 ml. of 10 per cent trichloroacetic acid solution. Treat the blank in the same way except that the fructose diphosphate is added after the reaction is stopped.

Centrifuge and to 1 ml. of supernatant liquid add 1 ml. of 3 per cent sodium hydroxide solution. Allow to stand for 10 minutes at room temperature and add 1 ml. of a filtered reagent prepared by dissolving 1 gram of 2,4-dinitrophenylhydrazine in 1 liter of 1:5 hydrochloric acid. Place in a 38° bath for 10 minutes. Precipitation at this stage is not significant. Add sufficient 3 per cent sodium hydroxide solution to bring the total to 10 ml. After 10 minutes read against the blank at 540 mμ. If the transmittence is less than 10 per cent, repeat the color development on a smaller aliquot of the trichloroacetic acid filtrate, adjusting to a volume of 1 ml. with 4.5 per cent trichloroacetic acid solution.

Amylase, Diastase

In the degradation of amylose by α-amylase, the enzyme attacks the polysaccharide chain to give hexose units of varying lengths.[4] Chains of 4-6 glucose units in length give no color with iodine, chains of 8-12 units give a red color, and chains of 30 units or longer give a blue color. This method is based on the blue color formed by the reaction of starch

[4] Marjorie A. Swanson, *Ibid.* 172, 805-14 (1948).

with iodine.[5] Measurements are made before and after incubation of soluble starch with material containing the enzyme. The decrease in color obtained after incubation is a measure of the amylase concentration. Starch-iodine color values obtained are proportional to the amount of enzyme present, with use of a fairly wide range of concentration of substrate. Instead of stopping the reaction with hydrochloric acid, picric acid is often used and is a reagent for reading the glucose formed.[6] As an alternative, after removal of unhydrolyzed starch, the glucose is developed by anthrone in strong acid.[7] The reaction of dextrin with diastase is also used for evaluation of enzyme action, the color of dextrin with iodine being used to determine the excess.[8] The removal of proteins by tungstic acid does not interfere. The ratio of the substrate hydrolyzed to the original concentration is a constant according to the equation

$$K = \frac{1}{0.4343t} \log \frac{Su_{t0}}{Su_t}$$

In this K equals the reaction constant, t equals reaction time in minutes, Su_{t0} equals amount of substrate at the start, and Su_t equals amount of substrate not hydrolyzed after t minutes. In practice the constant is represented as the value per minute from a table or calibration curve. The dextrin solution used as substrate must have been boiled to destroy diastase before dilution to volume. An 0.8 per cent solution of glycogen can replace a 0.5 per cent solution of dextrin in the method. The conversion of erythrodextrin to maltose as shown by the color with iodine is a relative measure of the amount of diastase in a body fluid.[9]

Using dextrin as the substrate, the diastatic activity may be measured

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[5] W. M. Persson, *Arch. intern. pharmacodynamie* **46**, 249-67 (1933); I. A. Remezov, *Arch. sci. biol.* (USSR) **37**, 425-31 (1935); Charles Huggins and Paul S. Russell, *Ann. Surg.* **128**, 668-78 (1948); Benjamin W. Smith and Joseph H. Roe, *J. Biol. Chem.* **179**, 53-9 (1949); E. P. Zinker and F. J. Reithel, *J. Lab. Clin. Med.* **34**, 1312-14 (1949); Eskil Hultin, *Acta Chem. Scand.* **3**, 886-94 (1949); D. Vincent and G. Segonzac, *Ann. biol. clin.* (Paris) **9**, 359-63 (1951); Cf. Vol. III, pages 225-233.

[6] Masamitu Tamai, *J. Biochem.* (Japan) **31**, 471-9 (1940); Victor C. Meyers, Alfred H. Free and Ethelreda E. Rosinski, *J. Biol. Chem.* **154**, 39-48 (1944).

[7] D. L. Morris, *Science* **107**, 254-5 (1948); Andre C. Kibrick, Hannah E. Rogers, and Sol Skupp, *J. Biol. Chem.* **190**, 107-10 (1951).

[8] O. Fischer, *Z. ges. exptl. Med.* **86**, 258-68 (1933); *Klin. Wochschr.* **15**, 791-2 (1936).

[9] I. Cohn, *Brit. Med. J.* **1924**, **I**, 618-20.

turbidimetrically in urine, serum, or plasma.[10]. The reducing sugars formed can also be measured by dinitrosalicylic acid [11] or with copper sulfate and sodium tungstate.[12]

Amylase activity in the pancreatic juice is determined by measurement of the turbidity at two stages during the hydrolytic action of the amylase on a standard starch suspension.[13] The reaction is monomolecular during the first five minutes. Accuracy is to about ±5 per cent. This reaction is also applicable with glycogen as the substrate with anthrone as the color reagent to react with the product of hydrolysis.[14]

The amylase unit is defined as the amount of enzyme which in the presence of 60 mg. of starch will hydrolyze 10 mg. of starch in 30 minutes to a stage at which no color is given with iodine at 620 mμ. The technic permits detection of amylase in concentrations up to 500 units per 100 ml.

Sample—*Serum.* Transfer two portions of 0.2 ml. of serum and 0.8 ml. of 1 per cent sodium chloride solution saturated with chloroform. Place in a water bath at 37° until the temperature of the bath is reached. Separately warm to the same temperature 3 ml. of a buffered dextrin solution which contains 5 parts of 0.5 per cent dextrin solution, 4 parts of 2.25 per cent monopotassium phosphate solution, 4 parts of 2.37 per cent anhydrous disodium phosphate solution, and 2 parts of 10 per cent sodium chloride solution. For storage, these reagents must be saturated with chloroform. After exactly 40 minutes remove one sample and stop enzyme action by adding at once 0.3 ml. of 1:6 hydrochloric acid. At the end of exactly 180 minutes similarly treat the other sample. To each add 0.2 ml. of 6 per cent sodium tungstate solution and mix. Centrifuge and decant the clear upper layer. Pipet 3.6 ml. of the decantate from each for development as dextrin by iodine.

As an alternative,[15] boil 0.1 ml. of sample, 0.3 ml. of phosphate buffer for pH 6 (Vol. I, page 176), and 2 ml. of 0.06 per cent starch solution in 0.9 per cent sodium chloride solution for 5 minutes. In the blank replace serum by buffer. Dilute to 50 ml. and incubate at 37°

[10] L. Jacobson and H. Hansen, *Scand. J. Clin. & Lab. Invest.*, **4**, 134-41, 183-8 (1952).

[11] Grant N. Smith and Cecelia Stocker, *Arch. Biochem.* **21**, 95-102 (1949).

[12] Joseph D. Teller, *J. Biol. Chem.* **185**, 701-4 (1950).

[13] Jerome M. Waldron, *J. Lab. Clin. Med.* **38**, 148-52 (1951).

[14] Harry Sobel and Sara M. Myers, *Ibid.* **41**, 655-8 (1953).

[15] Hugo Koch, *Deut. med. Wochschr.* **77**, 1481-2 (1952).

for 20 minutes. Add 1 ml. of 1 per cent sulfosalicylic acid solution and centrifuge. Complete by the last part of the procedure "By starch. Inactivation by hydrochloric acid" (page 492). Start at "Add 1 ml. of 0.3 per cent iodine . . . ."

For turbidimetric estimation mix 1 ml. of prewarmed serum or plasma with 1 ml. of the prewarmed buffer. Incubate at 38° for 30 minutes. Add 3 ml. of 8 per cent trichloroacetic acid, let stand for 10 minutes, and filter. To a 2-ml. aliquot of filtrate add 10 ml. of 95 per cent ethanol and let stand 50 minutes before reading turbidimetrically.

For estimation [16] by glycogen and anthrone prepare a substrate of 2 grams of glycogen, 11 ml. of a buffer containing 2.286 per cent of monopotassium phosphate, 1.551 per cent of disodium phosphate dodecahydrate, 2 per cent of sodium chloride, and 89 ml. of water. To 3 ml. of this at 37° add 0.2 ml. of serum and incubate for 15 minutes. Stop the reaction by addition of 3 ml. of absolute methanol. To a blank add the methanol and then the enzyme. Shake for 30 seconds, add 2 grams of Fuller's earth, shake for 2 minutes, and centrifuge.

Plasma. Prepare a buffer containing 2.286 per cent of monopotassium phosphate dodecahydrate and 2 per cent of sodium chloride. Dilute 11 ml. of buffer to 100 ml. as a base for the substrate. Heat 95 ml. of this to 90°. Disperse 1.25 grams of starch in 5 ml. of buffer and add to the 95 ml. at 90°. Let this cool to room temperature. The pH is about 7.1.

To 3 ml. of substrate at 37° add 0.5 ml. of plasma. After 30 minutes chill in ice water and add 3.5 ml. of 12.5 per cent trichloroacetic acid solution saturated with ammonium sulfate. At this time add 0.5 ml. of plasma to a control tube which had been processed up to this point. Add 0.5 gram of activated carbon and shake. Filter after 5 minutes. The filtrates give no color with iodine. Develop with anthrone.

Spinal fluid. To 1 ml. of spinal fluid add 3 ml. of buffered dextrin solution as described for use with serum. Add a few drops of chloroform to the substrate and sample and incubate at 37° for 12-15 hours, recording the time exactly. Stop activity by adding 0.3 ml. of 1:6 hydrochloric acid. Add 0.1 ml. of a suitable serum sample to provide an adequate amount of protein. Mix and add 0.1 ml. of 6 per cent sodium tungstate solution. Mix and centrifuge. If turbid add another 0.1 ml. of sodium tungstate solution, mix, and centrifuge again. Decant the

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[16] Harry Sobel and Sara M. Myers, *J. Lab. Clin. Med.* **41**, 655-8 (1953).

clear upper layer and pipet 3.6 ml. for development of dextrin by iodine.

Urine. Transfer three portions of 0.2 ml. of urine and 0.8 ml. of 1 per cent sodium chloride solution. Place in a water bath at 37°. Separately heat a portion of buffered dextrin solution as described under serum. When both have reached the temperature of the bath add 3 ml. of the dextrin solution to each sample. Mix and remove one tube after exactly 10 minutes, another after 40 minutes, and another after 160 minutes. Add 0.3 ml. of 1:6 hydrochloric acid immediately on removal, to stop enzyme action. If the urine is free of protein, add 0.2 ml. of distilled water to each. If the urine contains proteins, add 0.1 ml. of a suitable serum sample to each and mix. At once add 0.1 ml. of 6 per cent sodium tungstate solution, mix, and centrifuge. Then transfer 3.6 ml. from each as sample for development as dextrin by iodine.

For determination by glycogen and anthrone substitute urine for serum in the sample preparation described under the latter.

Procedure—*By starch. Inactivation by hydrochloric acid.* As a substrate, dilute 1.2 grams of soluble starch in 10 ml. of water to slightly under 100 ml. with boiling water. After heating at 100° for 3 minutes, dilute to volume at 90° and maintain at that temperature when pipeting.

Mix 5 ml. of starch solution, 3 ml. of a buffer containing 0.762 per cent of anhydrous potassium dihydrogen phosphate and 2.045 per cent of disodium hydrogen phosphate, and 1 ml. of 2.922 per cent sodium chloride solution. Prepare a duplicate as control and a blank omitting the starch. At 37° add 1 ml. of sample of serum, plasma, or urine. After exactly 30 minutes at 37° add 2 ml. of 1:10 hydrochloric acid to each tube. At the pH of below 2 amylase action will have ceased. Add 1 ml. of sample to the control and the blank.

Mix a 2-ml. aliquot of each with 400 ml. of water and 5 ml. of 1:10 hydrochloric acid. Add 1 ml. of 0.3 per cent iodine in 3 per cent potassium iodide solution to each and dilute to 500 ml. Read at 620 mμ against the blank. With D_c the control and D_s the sample digest, the result in mg. of starch destroyed = 60 $(D_c - D_s)/D_c$.

Inactivation by picric acid. Proceed as before through ". . . of serum, plasma, or urine." After exactly 30 minutes at 37° add 0.5 gram of dry solid picric acid. Agitate and filter. To 3 ml. of filtrate add 1 ml. of saturated sodium carbonate solution. Heat at 100° for 30 minutes, cool, and dilute to 20 ml. Read at 520 mμ against a reagent blank.

Dextrin by iodine. To each tube add 0.4 ml. of a solution containing

0.26 per cent of iodine and 0.52 per cent of potassium iodide and read against a natural standard.

Erythrodextrin by iodine. As buffer, dissolve 11.876 grams of disodium phosphate dihydrate in water and dilute to 1 liter. Dissolve 9.078 grams of anhydrous monopotassium phosphate in water and dilute to 1 liter. Mix 15 ml. of the disodium phosphate solution with 85 ml. of the monopotassium phosphate solution for use. Store the solutions under toluene in paraffined bottles.

As reagent add 20 ml. of 1.37 per cent iodine in potassium iodide solution to 500 ml. of saturated ammonium sulfate solution and dilute to 1 liter. Mix 1.5 ml. of sample with 6 ml. of the buffer solution. Mix 0.5 ml. of the mixture with 2 ml. of a solution containing 0.75 gram of erythrodextrin per 100 ml. Mix, stopper, and heat at 37° for one-half hour. When the sample solution is removed, cool at once. Add 15 ml. of the iodine reagent to sample, dilute to 30 ml. and read.

By glycogen and anthrone. Dilute 0.4 ml. of prepared sample to 2 ml. with water. Add 4 ml. of a reagent containing 2 mg. of anthrone per ml. in 95 per cent sulfuric acid. Read against the blank. Results can be read from a glucose curve.

By starch and anthrone. To 2 ml. of sample add 3 ml. of water and 10 ml. of 0.2 per cent solution of anthrone in 92 per cent sulfuric acid. After cooling for 15 minutes read against water and correct for the blank and control.

By turbidity. Pancreatic juice. As substrate mix 0.5 ml. of a commercial 10 per cent starch suspension containing 4 per cent of sodium chloride and 0.5 per cent of pine oil to prevent bacterial deterioration; add 1 ml. of 3.6 per cent sodium chloride solution and 3.5 ml. of a phosphate buffer for pH 7.2 (Vol. I, page 176). Warm to 38° and shake.

Shake the warm substrate, add 1 ml. of 1:100 pancreatic juice in a phosphate buffer for 7.2, and time with a stopwatch. Shake and warm for 1 minute at 38° and then read the turbidity at 420 mμ. Replace in the bath and read the turbidity again after exactly 3 minutes. Read against a water blank, taking 1 ml. of 1:100 pancreatic juice as representing 100 per cent on the curve. The velocity constant is:

$$k = 1.15 \log C_1/C_2$$

Duodenal contents. When duodenal contents are used as sample, prepare a blank by diluting 1 ml. of the 1:100 duodenal contents, with

5 ml. of phosphate buffer. Subtract the turbidity readings of this at 1 minute and 3 minutes from the reading of the sample developed as described for pancreatic juice.

Urine, serum, or plasma. Add 0.1 ml. of sample to 1 ml. of pre-warmed buffer at 38°. Incubate for 30 minutes and add 10 ml. of 72 per cent ethanol. Let stand at room temperature for 40 minutes before reading at 720 mμ against distilled water.

Arginase

Arginase acts on arginine to give an amount of urea molecularly proportional to the amount of enzyme.[17] The urea is then reacted with α-nitrosopropiophenone to give the color for reading.[18] An alternative is α-naphthol.[19] Urease must be absent as it will liberate carbon dioxide from urea. The action of arginase on arginine is inhibited by the presence of ornithine, but if the concentration of arginine is large compared to that of ornithine, retardation is insignificant. A unit of arginase activity is defined as that amount which in 1 minute at 25° and pH 9.5 with 0.285 *M* substrate will decompose 1 micromole of arginine to form 1 micromole of urea.

Procedure—As arginine solution dissolve 9 grams of arginine monohydrochloride in water containing 1.6 ml. of 72 per cent sodium hydroxide solution. Protect from absorption of carbon dioxide.

Protein greater than 0.2 mg. per ml. Immediately before the analysis dilute the arginase solution so that it contains 0.1-0.4 arginase unit per ml. Bring both arginine and arginase to measured room temperature. Adjust the arginine solution to pH 9.5 and, to 0.5 ml., add 1 ml. of freshly diluted arginase solution. Mix the substrate and enzyme immediately. Exactly 10 minutes after the arginine and arginase have been mixed, stop the action by adding 1 ml. of 15 per cent metaphosphoric acid. After 15 minutes to deproteinize, centrifuge and decant. To 2 ml. of decantate add 15 ml. of a solution prepared by mixing 90 ml. of concentrated sulfuric acid and 270 ml. of sirupy phosphoric acid with water and dilution to 1 liter.

To 10 ml. of this solution, add 0.5 ml. of 3 per cent α-isonitrosopro-

[17] Donald D. Van Slyke and Reginald M. Archibald, *J. Biol. Chem.* **165**, 293-309 (1946).

[18] Reginald M. Archibald, *Ibid.* **157**, 507-18 (1945).

[19] S. N. Iyer and C. R. Krishna Murti, *Experientia* **8**, 308-9 (1952).

piophenone solution in ethanol and mix. Stopper and heat for 1 hour in boiling water. Cool in the dark and thereafter protect from light. Read at 540 mμ against a reagent blank. The calculation is based on the following:

$$E = 0.212UC$$

where U = observed optical density of the unknown
E = arginase content in units/ml.
C is obtained from Table 14

TABLE 14. FACTORS TO GIVE MICROMOLES OF ARGININE DECOMPOSED BY SAMPLE AT 25°

Temperature (° C.)	*C*	*Temperature (° C.)*	*C*
10	2.50	25	1.00
11	32	26	0.95
12	17	27	90
13	03	28	85
14	1.92	29	81
15	82	30	77
16	72	31	73
17	62	32	69
18	52	33	66
19	42	34	635
20	33	35	610
21	25	36	585
22	17	37	565
23	10	38	545
24	05	39	522

Protein in less than 0.2 mg. per ml. Proceed as for larger amounts of protein through ". . . stop the action." Add 20 ml. of the phosphoric-sulfuric acid mixture for each 1.5 ml. of digest. Complete from "To 10 ml. of this solution . . .." $E = 0.215UC$.

CATALASE

Catalase activity is estimated by allowing the enzyme solution to react with hydrogen peroxide for varying periods of time, adding excess potassium permanganate, and reading the amount remaining.[20]

[20] Samuel A. Goldblith and Bernard E. Proctor, *J. Biol. Chem.* **187**, 705-9 (1950).

Procedure—As substrate dilute 0.15 ml. of 30 per cent hydrogen peroxide with 25 ml. of 0.05 *M* phosphate buffer for pH 7 (Vol. I, page 176). To 2 ml. of catalase preparation buffered at pH 7 with 0.05 *M* phosphate buffer in the cuvet add 1 ml. of buffered substrate at zero time. Read at 240 mμ every ten seconds until at least half the substrate is destroyed. Use a blank for the setting. Plot the values which should give a straight line. Alternatively read with a stopwatch the time when half the substrate is destroyed. Either method gives the velocity and therefore the activity of the catalase.

Carbonic Anhydrase

The concentration of carbonic anhydrase is determined by the effect of pH change on bromothymol blue.[21] The method is to measure the time required for the pH to drop from approximately pH 8 to 6.3. Veronal is an appropriate buffer.

Procedure—Draw 2 ml. of saturated carbon dioxide solution into a cold syringe and immerse in a water-ice mixture until needed. As buffer dissolve 4.536 grams of sodium barbiturate in about 950 ml. of water, adjust to pH 8.15 with barbituric acid, and dilute to 1 liter. Protect from carbon dioxide.

Mix 2 ml. of cold buffer containing 0.05 mg. of bromothymol blue per ml. and 1 ml. of the sample solution. Stopper and chill in an ice-water mixture. Rapidly eject the carbon dioxide solution into the tube containing the buffer solution and determine the end point when the color matches the buffer at pH 6.3.

Carboxypeptidase

Carboxypeptidase attacks peptide bonds involving the α-amino group of *l*-phenylalanine, *l*-tyrosine, or *l*-tryptophan.[22] Substrates such as carbonaphthoxyphenylalanine upon hydrolysis decarboxylate immediately to give β-naphthol, which in turn can be coupled with a suitable diazonium salt to yield an insoluble azo dye. The azo dye formed of

[21] R. Brinkman, *J. Physiol.* **80**, 170-3 (1933); F. J. and J. St. L. Philpot, *Biochem. J.* **30**, 2191-3 (1936); H. van Goor, *Entomologia* **8**, 113-28 (1940); D. Keilin and T. Mann, *Biochem. J.* **34**, 1163-76 (1940); F. J. W. Roughton and V. H. Booth, *Ibid.* **40**, 319-30 (1946); Karl M. Wilbur and Norman G. Anderson, *J. Biol. Chem.* **176**, 147-54 (1948).

[22] Herbert A. Ravin and Arnold M. Seligman, *Ibid.* **190**, 391-402 (1951).

tetrazotized diorthoanisidine and 2 molecules of β-naphthol is extracted into ethyl acetate and read. An alternative is to follow the hydrolysis of carbobenzoxyglycyl-*l*-phenylalanine by the ninhydrin reaction [23] (page 107).

Sample—*Tissue.* Disperse fresh tissue in 0.85 per cent sodium chloride solution in a concentration of 1-2 mg. per ml. and allow to settle 2-3 minutes for use of the supernatant liquid.

Procedure—Dissolve 0.04 gram of carbonaphthoxyphenylalanine in 2 ml. of acetone and dilute to 100 ml. Mix 1 ml. of sample, 1 ml. of a solution containing 0.1 mg. of trypsin per ml., and 3 ml. of 1 per cent sodium barbital adjusted to pH 7.8 with barbituric acid. The trypsin converts enzymatically inactive procarboxypeptidase to carboxypeptidase. After 3 minutes, add 1 ml. of freshly prepared stock solution of substrate and incubate at 37° for 60 minutes. Add 1 ml. of solution of tetrazotized diorthoanisidine, Du Pont Naphthanil diazo blue B, containing 4 mg. per ml., and allow coupling to go on for 3 minutes. Add 1 ml. of 80 per cent trichloroacetic acid solution to inhibit further hydrolysis of the substrate and extract into 10 ml. of ethyl acetate. Read at 540 mμ against a β-naphthol curve.

Cathepsin

Cathepsin is estimated nephelometrically by hydrolysis of a sodium caseinate substrate followed by nephelometric estimation of residual casein by quinidine hydrochloride.[24] Optimum results are obtained at pH 4-5, which is near the isoelectric point of casein, with accuracy to ±5 per cent. An alternative is hydrolysis of edestin with thiosalicylic acid as a nephelometric reagent for estimation of the amount not hydrolyzed.[25] Accuracy to ±9 per cent is obtainable.

Sample—*Spleen.* Cut up the fresh spleen, mix with 5 parts by weight of a solution containing 89 per cent of glycerol and 0.15 per cent of acetic acid. Keep this crude extract in a refrigerator with added toluene as preservative. Before use dilute with 9 parts of water and centrifuge.

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[23] George W. Schwert, *Ibid.* **174**, 411-13 (1948).

[24] W. N. Nicholson and D. Rhind, *Analyst* **49**, 505-9 (1924).

[25] K. G. Stern, *Biochem. Z.* **236**, 464-73 (1931); B. J. Krijgsman, *Z. physiol. Chem.* **228**, 256-67 (1934).

Normal tissue and tumors. Free the sample from fat and blood, wash with 0.9 per cent sodium chloride solution, and grind. Weigh a sample and mix with twice its weight of 37 per cent glycerol solution to which 0.15 per cent of acetic acid has been added. Shake for 4 hours at 37° and filter.

Procedure—*By sodium caseinate.* Mix 0.84 ml. of the sodium caseinate solution as prepared for the nephelometric method for trypsin (page 531), 1.05 ml. of a buffer containing 1 part of 0.92 per cent monosodium phosphate monohydrate solution to 19 parts of 2.39 per cent disodium phosphate dodecahydrate solution, and 1.33 ml. of distilled water. Transfer two 1.4-ml. samples of this mixture. To one add 0.1 ml. of sample solution. To the other add 1 ml. of the sample solution which has been heated in boiling water until inactive and cooled. Pipet 0.3 ml. from the sample and sample blank into 0.3 ml. portions of boiling water, and heat in boiling water for exactly 6 minutes. Stopper the sample and that containing the mixture of cathepsin and substrate with cotton wet with toluene and place in a thermostat at 37°. Remove further 0.3-ml. portions from the tubes from time to time. To develop the turbidity add 0.3 ml. of a buffer containing 1 part of the specified monosodium phosphate solution and 9 parts of the disodium phosphate solution and 0.3 ml. of saturated quinidine hydrochloride reagent. After 10 minutes read nephelometrically.

By edestin. As substrate dissolve 1 gram of edestin by boiling with 1 liter of 1:1200 hydrochloric acid and filter. Add a few drops of toluene and keep in a refrigerator.

Mix 2.5 ml. of edestin substrate with 0.63 ml. of 6.976 per cent veronal acetate solution, 1.38 ml. of 1:120 hydrochloric acid, and 1.1 ml. of water. Transfer two 2.3-ml. portions and add 0.2 ml. of sample cathepsin solution to one and 0.2 ml. of the sample solution which has been rendered inactive by heating to the other. Mix each and pipet 0.3 ml. into 0.3 ml. of boiling water. Heat each of these for exactly 6 minutes in boiling water and set aside. Stopper the sample and control with cotton saturated with toluene and place them in a thermostat at 35°. Remove further 0.3-ml. portions of sample and sample blank from time to time, add to 0.3 ml. of boiling water, and heat as previously described. To develop the turbidity of the samples and controls, add 0.75 ml. of 0.4 per cent sodium hydroxide solution and 0.24 ml. of 10 per cent thiosalicylic acid solution, and mix. Compare the sample with the sample

blank, to estimate the degree of hydrolysis by cathepsin. The turbidity lasts only about 10 minutes.

Cholinesterase and Pseudocholinesterase

The two forms of cholinesterase are determined by the same method, their effect in hydrolyzing phenyl benzoate followed by measurement of the phenol liberated.[26] Hydrolysis is proportional to time [27] for at least 2 hours, provided not more than 0.028 mg. of phenol in a 5-ml. sample is liberated.

Hydrolysis of carbonaphthoxycholine iodide as catalyzed by cholinesterase is read in terms of β-naphthol coupled with tetrazotized diorthoanisidine.[28] A unit is the amount of enzyme which liberates 10 mg. of β-naphthol per hour from β-naphthyl acetate. Upon reaction with acetic acid, dilute protein solutions become instantaneously opaque in direct proportion to the amount of the acid.[29] By use of this observation, cholinesterase activity is measured with an accuracy of 3-5 per cent. The change in pH is also a measure of the activity.[30] Acetylsalicylic acid is a substrate which can be read as the hydrolytic product, salicylic acid, at 290-300 mμ.[31]

Another method of estimation is by direct hydrolysis of acetylcholine. The change in acidity as a function of pH is measured [32] with a buffer to control the pH change of the liberated acetic acid within the limits of pH 6-8. As an alternative unhydrolyzed acetylcholine is converted by hydroxylamine in alkaline solution to acethydroxamic acid which is developed with ferric ion.[33] Results in terms of micromoles of acetyl-

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26 J. Alfred Rider, Hugo C. Moeller, and Kenneth P. DuBois, *Proc. Soc. Exptl. Biol. Med.* **76**, 427-9 (1951).

27 G. Gomori, *J. Lab. Clin. Med.* **34**, 275-81 (1949).

28 Herbert A. Ravin, Kwan-Chung Tson, and Arnold M. Seligman, *J. Biol. Chem.* **191**, 843-57 (1951).

29 I. Gal, *Ann. biol. clin.* (Paris) **6**, 363-5 (1948).

30 H. Croxatto, R. Croxatto, and F. Huidobro, *Anales acad. biol.* Univ. Chile **3**, 55-65 (1939); Hiroshi Takahashi and Susumu Shibata, *Igaku to Seibutsugaku* **20**, 96-8 (1951).

31 B. H. J. Hofstee, *Science* **114**, 128-30 (1951).

32 Harry A. Michel, *J. Lab. Clin. Med.*, **34**, 1564-8 (1949); J. Gregoire and M. Cotte, *Compt. rend. soc. biol.*, **146**, 741-4 (1952); John J. Reingold, Licia Gambescia Tourigny, and Virginia L. Yonan, *Am. J. Clin. Pathol.* **23**, 645-53 (1953).

33 Robert L. Metcalf, *J. Econ. Entomol.*, **44**, 883-90 (1951); J. De la Huerga, Charlotte Yesinick, and Hans Popper, *Am. J. Clin. Path.* **22**, 1126-33 (1952).

choline bromide hydrolyzed per hour per ml. of serum are normally 130-310.

Sample—*Serum.* Dilute 1:100 or 1:200 for use as sample, according to the concentration anticipated. Determine by phenyl benzoate. For estimation by acetylcholine either by reading the pH or as acethydroxamic acid use the serum undiluted.

Gastric juice. Dilute 1:25 and determine by phenyl benzoate.

Procedure—*By phenyl benzoate.* Prepare a buffered substrate by adding 1 ml. of phenyl benzoate solution containing 2 grams in 100 ml. of methanol, to 500 ml. of a phosphate buffer for pH 6.3. This contains 0.535 per cent of disodium phosphate dodecahydrate and 0.7 per cent of monosodium phosphate monohydrate. Stir the buffered substrate and keep in a refrigerator. To prepare a diazo reagent, dissolve 0.25 gram of Du Pont Naphthanil Diazo Red B salt in 100 ml. of ice-cold water and filter.

To 5 ml. of prewarmed buffered substrate add 1 ml. of sample solution containing esterase. Incubate at 37° for 1 hour and then place in ice-cold water to stop esterase activity. As a blank add 1 ml. of sample solution to prewarmed buffered substrate but do not incubate. To sample and blank add 4 ml. of a 3.5 per cent borax decahydrate solution in 15 per cent ethanol. Add 0.5 ml. of the diazo reagent. Mix and read after 10 minutes against the blank, at 500 mμ.

By β-naphthol. As a buffer for pH 7.4, shortly before use mix 27.5 ml. of 2.06 per cent solution of sodium diethylbarbiturate with 20 ml. of 1:160 hydrochloric acid. As substrate dissolve 21.6 mg. of carbonaphthoxycholine iodide in 5 ml. of acetone and dilute to 100 ml. with water. This keeps for a week at 4°.

Mix 0.1 ml. of serum with 16 ml. of water. Mix 5 ml. of a 1:1 mixture of buffer and substrate with 1 ml. of the diluted serum. Incubate at 37° for 1 hour. Add 1 ml. of fresh 0.2 per cent aqueous tetrazotized diorthoanisidine. This is conveniently prepared from Du Pont Naphthanil Diazo Blue B which contains 20 per cent of this agent. Mix and, after 3 minutes for coupling, add 1 ml. of 80 per cent trichloroacetic acid to stop reaction. Extract the dye with 10 ml. of ethyl acetate and read at 540 mμ against a blank to which the serum was not added. Read against a β-naphthol curve.

By acetylcholine. Milk. Mix 0.3 ml. of serum, 0.3 ml. of milk, 0.1 ml. of 0.1816 per cent acetylcholine chloride solution, and 1.1 ml

of water. Read the opacity at 3-minute intervals and plot. From this determine when half of the acetylcholine has been hydrolyzed. Express cholinesterase activity as the reciprocal of this time multiplied by 1,000.

By acidity from acetylcholine. For reading in terms of hydrolysis of acetylcholine first prepare a buffer for pH 8. The concentrate contains 2.474 grams of sodium barbital, 0.272 gram of monopotassium phosphate, and 35.07 grams of sodium chloride per liter. For use dilute 250 ml. of this and 5.8 ml. of 0.1 *N* hydrochloric acid to a liter.

Add 0.1 ml. of serum to 10 ml. of the diluted buffer. Place 2-ml. portions in cuvets at 25° for 5 minutes. Add 0.2 ml. of 0.01 per cent phenol red solution to one and an equal amount of water to another. Add 0.2 ml. of 3 per cent aqueous acetylcholine chloride to each. Read at 540 mμ. Incubate at 25° for 60 minutes and read again.

By acetylcholine and hydroxlyamine. As buffer-reagent prepare the following. First dissolve 10.3 grams of sodium barbital in 300 ml. of water and add 60 ml. of *N*-hydrochloric acid. Add 5.3 grams of sodium carbonate and dilute to 500 ml. Mix 8 volumes of this buffer, 1 volume of a salt solution containing 4.2 per cent of magnesium chloride and 0.2 per cent of potassium chloride, and 1 volume of 11.3 per cent acetylcholine bromide.

Warm 2-ml. portions of the buffer-reagent to 37°. To one add 0.2 ml. of serum; to the other, 0.2 ml. of water. Incubate for 1 hour at 37° and add 2 ml. of a 1:1 mixture of 14 per cent hydroxylamine hydrochloride solution and 14 per cent sodium hydroxide solution. After 1 minute add 6 ml. of 1:19 hydrochloric acid. To 0.5 ml. of each add 10 ml. of 1 per cent solution of ferric chloride hexahydrate and centrifuge. Read at 540 mμ and interpret in terms of an acetylcholine curve.

Coenzyme A

Coenzyme A is measured by the rate of reduction of diphosphopyridine nucleotide by α-ketoglutarate, catalyzed by a soluble oxidase and coenzyme A. The latter is continually regenerated from the succinyl coenzyme A formed in the reaction by means of a succinyl coenzyme A deacylase.[34] Adenosine triphosphate, adenosine diphosphate, inorganic pyrophosphate, and copper inhibit the reaction. Pyridine does not interfere at 0.3 per cent or less. The method is applicable to 0.5-1.5 Lipmann units of coenzyme A.

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[34] R. W. Von Korff, *J. Biol. Chem.* **200**, 401-5 (1953).

Sample—To 0.1 ml. of 0.158 per cent cysteine hydrochloride neutralized to pH 7 with potassium hydroxide solution, add 0.1 ml. of coenzyme A solution of pH 7 containing 5-15 units per ml., 0.1 ml. of 13 per cent potassium glycinate solution at pH 9, 0.1 ml. of 2.92 per cent α-ketoglutarate at pH 7, 0.1 ml. of deacylase, 0.01 ml. of α-ketoglutaric oxidase, and water to 2.9 ml. With each pair of samples, treat a reference solution of coenzyme A in the same manner. Mix each and incubate for 5 minutes at 30°. Read against water at 340 mμ.

Procedure—Add 0.1 ml. of a 1 per cent solution of diphosphopyridine nucleotide at pH 7 to each tube. Mix and read at 340 mμ at 1-minute intervals for 3-5 minutes, with a 10-second interval between tubes. The amount of coenzyme A in the sample is proportional to the average decrease in absorption per minute.

Cozymase

Cozymase is assayed by reduction of the diphosphopyridine nucleotide content to dihydrocozymase and read at 340 mμ.[35]

Procedure—To a sample solution containing approximately 10 mg. of cozymase, add 2 ml. of fresh 0.2 per cent sodium hydrosulfite in 1 per cent sodium bicarbonate solution. Immerse in boiling water at once for 1 minute and chill. During the heating, yellow due to monohydrocozymase fades. Further dilute during cooling with a buffer for pH 9.7 containing 1 per cent each of sodium bicarbonate and sodium carbonate. Dilute to 50 ml. and aerate a portion until the hydrosulfite is oxidized. Usually 5 minutes are required. Read at 340 mμ against the buffer solution, aerate for 3 minutes longer, and read again at 340 mμ. Also read the reduced solution. When the reading of the aerated solution is constant apply the difference against a known curve.

Cytochrome Oxidase

Cytochromes differ from hemoglobin in that the protein component is not linked alone by partial valencies to the iron. The methods used for colorimetric determination of phenol oxidase and peroxidase are applicable to determination of cytochrome oxidase in animal tissues.[36]

[35] Sidney Gutcho and Earl D. Stewart, *Anal. Chem.* **20**, 1185-7 (1948).

[36] H. G. Albaum, J. Tepperman, and O. Bodansky, *J. Biol. Chem.* **163**, 641-7 (1946); Frederick G. Smith and Elmer Stotz, *Ibid.* **179**, 891-902 (1949).

Any substances in the enzyme preparation capable of reducing the oxidized dye can cause lower oxidation rates in the oxidase assay. Reducing materials in the homogenate are detected by measuring dye reduction in a system consisting of buffer, oxidized dye, and enzyme. An alternative technic is by comparative evaluation against reduced solution.[37]

Sample—*Tissue.* After surgical removal of tissues[38] from freshly killed animals or humans, store in ice-cooled jars in the refrigerator until used. Blot dry and weigh samples of 0.5-1 gram of tissue. Cut into small pieces and homogenize in 5-10 ml. of water in a water bath. Keep the homogenate in an ice bath and assay as soon as possible.

Procedure—*By leuco-2,6-dichlorobenzeneoneindo-3′-chlorophenol.* Prepare a solution of 2,6-dichlorobenzeneoneindo-3′-chlorophenol containing 0.035 per cent every 3-4 days. Suspend about 0.5 mg. of 5 per cent palladized asbestos in 20-30 ml. of this solution by shaking. Add 4 drops of 0.2 *M* buffer for pH 6 (Vol. I, page 176) and bubble hydrogen through the solution until it is decolorized. Filter with suction and resume a slow stream of hydrogen to prevent autoxidation.

Dilute the oxidase sample immediately before assay. Keep all reagents at 30°. Mix 1 ml. of 0.2 *M* phosphate buffer for pH 7 (Vol. I, page 176), 0.5 ml. of sample solution, 1 ml. of the reduced dye, and 0.5 ml. of 0.32 per cent cytochrome C solution. Swirl quickly to mix and read at 645 mμ at 5-second intervals in the period from 15-45 seconds after adding the enzyme. Subtract a reagent blank and calculate the rate of reaction from the flat part of the curve.

Against reduced solution. To 30 ml. of 0.26 per cent solution of cytochrome C in 0.03 *M* phosphate buffer for pH 7.4 (Vol. I, page 176), add 0.1 ml. of fresh 25 per cent sodium hydrosulfite solution. Shake vigorously for 2 minutes to destroy excess hydrosulfite.

To 3 ml. of reduced cytochrome C solution add 0.02 or 0.04 ml. of sample solution, mix, and read every 30 seconds at 550 mμ. After 3 minutes add a few grains of potassium ferricyanide to oxidize the remaining cytochrome C and read the background color.

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[37] S. J. Cooperstein and Arnold Lazarow, *Ibid.* **189**, 665-70 (1951).

[38] Otto Rosenthal and David L. Drabkin, *Ibid.* **149**, 437-50 (1943).

DEHYDROPEPTIDASE

Dehydropeptidase in tissue extracts splits the peptides of α-aminoacrylic acid to yield products including ammonia and pyruvic acid.[39] Activity is estimated by noting the rate of formation of these products from glyceryldehydroalanine as read by the decrease in ultraviolet absorption.

Procedure—*Tissue.* Grind the tissue with sand and extract with 12 volumes of water. Centrifuge and to 0.2 ml. add 0.2 ml. of 0.36 per cent glycyldehydroalanine solution. Incubate for various lengths of time at 37°, add 10 ml. of water, and read at 250 mμ against a sample blank. Plot the rate of decrease and from a linear part of the curve express it in mg. of substrate split per hour per mg. of total nitrogen in the extract.

EREPSIN

The dipeptidase activity of erepsin is estimated by its action on glycylglycine. For maximum activity the solution is adjusted to pH 8. As hydrolysis proceeds the glycine liberated is less acid than glycylglycine. The pH therefore rises and the extent of hydrolysis is estimated by colorimetric pH methods, using phenolphthalein as indicator.[40] The pH must be adjusted with sodium carbonate solution because phosphate buffers lower the enzyme activity.

Sample—*Pig intestines.* Free the mucous membrane of pig intestines from foreign matter. Wash with distilled water and cut fine. Rub in a mortar with 83.7 per cent glycerol. Let this stand for a day and dilute with 3 volumes of water. Centrifuge to give a clear, light yellow, opalescent solution.

Procedure—To 10 ml. of 0.264 per cent glycylglycine solution add 6 drops of 10.6 per cent sodium carbonate solution. To 10 ml. of the

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[39] M. Bergmann and H. Schleich, *Z. physiol. Chem.* **205**, 65 (1932); *Ibid.* **207**, 235 (1932); J. P. Greenstein and F. M. Leuthardt, *J. Biol. Chem.* **162**, 175-6 (1946); *J. Nat. Cancer Inst.* **6**, 197 (1946); Charles E. Carter and Jesse P. Greenstein, *J. Biol. Chem.* **165**, 727-8 (1946).

[40] E. Waldschmidt-Leitz, A. K. Balls and J. Waldschmidt-Graser, *Ber.* **62**, 956-62 (1929); E. Waldschmidt-Leitz and A. Purr, *Ibid.* **62**, 2217-26 (1929); W. M. Persson, *Arch. intern. pharmacodynamie* **49**, 204-11 (1934).

erepsin sample add 2 drops of 4 per cent sodium carbonate solution. Mix 10 ml. of the glycylglycine solution, 3 ml. of water, and 3 ml. of the erepsin sample. To study the rate of enzyme action prepare a series of such dilutions. Place one in a thermostat at 45°. At the end of each 10-minute period for 2 hours introduce another similar tube. At the end of the 2-hour period cool in water. Add 1 drop of 1 per cent phenolphthalein solution to each and read at 550 mμ.

Esterase

Esterase liberates acetic acid from acetylcholine. This gives a brownish-red color with ferric chloride.[41] The reaction is also given by other choline esters. Esterase liberates β-naphthol from β-naphthyl laurate by a technic implicit in the method for lipase (page 510). Another technic is by hydrolysis of fatty acid esters of salicylic acid.[42] The buffered solution is read at 300 mμ where the free salicylic acid absorbs and the ester does not. Another is by liberation of phenolphthalein from phenolphthalein dibutyrate.[43] The liberated indicator is read in alkaline solution.

Procedure—*Blood plasma.* To 10 ml. of a buffer solution, containing 5 volumes of 3.81 per cent borax solution and 15 volumes of 2.47 per cent boric acid solution, add 5 ml. of 1:20 plasma and 10 ml. of 0.756 per cent acetylcholine chloride solution. Stopper tightly and place in a 37° bath for about 200 minutes. Cool under running water and add 2.5 ml. of 15 per cent ferric chloride solution. Read at 570 mμ and calculate the rate of hydrolysis.

β-D-Galactosidase

β-Galactosidase hydrolyzes β-D-galactosides, α-L-arabinosides, lactose, and heptosides with a β-D-galactose configuration. It occurs with or without β-glucosidase. An appropriate colorimetric method is by hydrolysis of 6-bromo-2-naphthyl-β-D-galactopyranoside which reacts 10 times as fast as the unbrominated compound.[44] Dyes produced from the

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41 N. O. Obdon and B. Uvnas, *Skand. Arch. Physiol.* **76**, 1-14 (1937).

42 B. H. J. Hofstee, *Science* **114**, 128-30 (1951).

43 Arnuff Purr, *Biochem. Z.* **322**, 205-11 (1951).

44 Richard B. Cohen, Kwan-Chung Tsou, Selma H. Rutenberg, and Arnold M. Seligman, *J. Biol. Chem.* **195**, 239-49 (1952).

β-naphthols are more easily extracted than those from α-naphthol. Tetrazotized diorthoanisidine is preferred. Iron, zinc, mercury, and magnesium salts interfere with the action of the enzyme, as do also diazonium salts and fixing agents such as acetone, formalin, etc. The method is accurate to about ±5 per cent.

Sample—As substrate heat 100 grams of D-galactose, 540 ml. of acetic anhydride, and 44 grams of anhydrous sodium acetate at 100° for 20 minutes with stirring. Cool, pour into 1600 ml. of ice water, and stir for 1 hour. Extract with benzene, wash, dry with anhydrous calcium sulfate, and evaporate to a sirup from which the penta-acetate crystallizes. Recrystallize from alcohol to give a 55 per cent yield of penta-acetate melting at 142-3°.

Deacetylate by dissolving 3 grams of the penta-acetate in 30 ml. of methanol and treating with a piece of sodium 2-3 mm. in diameter. Let stand at 4° overnight. Recrystallize the product from methanol to give an 85 per cent yield of 6-bromo-2-naphthyl-β-D-galactopyranoside melting at 205°.

To buffer this, dissolve 15 mg. in 10 ml. of boiling absolute methanol plus 15 ml. of water. Add 55 ml. of water and 20 ml. of a phosphate-citrate buffer for pH 4.95 containing equal volumes of 1.92 per cent citric acid solution and 1.42 per cent anhydrous disodium phosphate solution, preserved with 0.2 per cent of toluene by volume.

Tissue. Homogenize tissue from freshly killed animals or from humans as soon after death as possible, at a ratio of 5 mg. per ml. of water, with the aid of a mechanical ground-glass homogenizer, for 2 minutes. Centrifuge and use the supernatant liquid as sample. The enzyme activity remains stable for several days at 4°.

Procedure—To 0.6 ml. of prepared enzyme sample add 5 ml. of buffered substrate and incubate at 37° for 2 hours. As control, use 0.6 ml. of boiled sample plus 5 ml. of buffered substrate. Add 0.5 ml. of 7.6 per cent trisodium phosphate dodecahydrate solution to bring the pH to 7.5-7.8, and 1 ml. of cold diazotization reagent containing 1 mg. per ml. of Du Pont Naphthanil Diazo Blue B. Mix for a minute, add 2 ml. of 80 per cent trichloroacetic acid to precipitate protein, and release the dye. Add 10 ml. of chloroform, shake, and let stand. Remove about 5 ml. of the chloroform layer containing the dye and centrifuge. Read at 540 mμ against the control.

β-D-Glucosidase

This enzyme is determined by its hydrolytic effect on 6-bromo-2-naphthyl-β-D-glucopyranoside. It cleaves the β-D-glucosidic linkage of simple alkyl and aryl glucosides. After action by the enzyme, the 6-bromo-2-naphthol liberated is treated to form an azo dye for reading.[45] The method is the same as that for β-D-galactosidase, and all of the same conditions and comments apply, except for use of a different substrate and increase of the incubation period from 2 to 5 hours.

Sample—As substrate, fuse 10 grams of β-D-glucopyranose pentacetate with 16 grams of 6-bromo-2-naphthol, and 50 mg. of *p*-toluene-sulfonic acid *in vacuo* for 30 minutes at 100°. Take up with 100 ml. of benzene and wash with ice-cold water, with 2 per cent sodium hydroxide solution, again with water, and dry over anhydrous calcium sulfate. Evaporate under reduced pressure and recrystallize from methanol to give a 75 per cent yield of 6-bromo-2-naphthyl-β-D-tetraacetyl glucopyranoside melting at 142-143°.

Deacetylate by suspending 3 grams of this in anhydrous methanol and adding a piece of sodium 2-3 mm. in diameter. Let stand at 4° for 2 days, to give 80-85 per cent yield of 6-bromo-2-naphthyl-β-D-glucopyranoside melting at 206-207°.

To buffer this, dissolve 10 mg. in 20 ml. of boiling absolute methanol plus 20 ml. of water. Add 20 ml. of phosphate-citrate buffer for pH 4.95 (Vol. I, page 176) and 40 ml. of water. Use immediately.

Tissue. Prepare as for determination of β-D-galactosidase.

Procedure—Treat the same as for β-D-galactosidase, starting "To 0.6 ml. of prepared enzyme sample add 5 ml. of buffered substrate . . ." except to incubate at 37° for 5 hours instead of 2 hours.

Histaminase

Histaminase at pH 7.2 and 38° is estimated by the unreacted histamine at the end of 24 hours of agitation. A suitable reagent is diazotized sulfanilic acid.[46]

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[45] Richard B. Cohen, Selma H. Rutenberg, Kwan-Chung Tsou, Michael A. Woodbury, and Arnold M. Seligman, *J. Biol. Chem.* **195**, 607-14 (1952).

[46] Kijooki Takahasi and Tosio Umeda, *J. Agr. Chem. Soc. Japan* **15**, 939-46 (1939).

Hyaluronidase

Activity of the enzyme, hyaluronidase, is measured by determining the amount of protein combined with hyaluronate substrate. The protein is estimated by combination with the dye, disodium phenoltetrabromophthalein (bromosulfalein) and then dissolving the complex in alkaline solution.[47] The method is accurate to about ±10 per cent.

Substrate.[48] Thaw human umbilical cords which have been stored at −20°, strip manually, and grind. Mix with an equal volume of water and place in an Arnold sterilizer. After 30 minutes at 100° let stand 12 hours at 0°. Strain through cheese-cloth and again heat at 100° for 30 minutes. Chill to 0° and centrifuge off the brown precipitate to give a cloudy gray fluid. Add 20 grams of Filter-Cel per liter and filter on a Büchner funnel. Pour the clear straw-to-pink filtrate into 4 volumes of cold acetone without stirring. Remove the stringy white-to-brown floating material. Wash with acetone and then with ether, evaporate the ether, and dry over calcium chloride *in vacuo*. Grind to a fluffy powder for use.

Sample—Prepare a solution of hyaluronidase from the dried commercial product or from physiological material, to contain 0.27 mg. of nitrogen per ml. For dilution of the enzyme to this concentration use an acetate buffer for pH 6 containing 0.82 per cent of sodium acetate and 0.88 per cent of sodium chloride. Transfer 0.5 ml. of enzyme solution buffered to pH 6 into each of 5 tubes and warm to 37.5°. Prepare a solution of the prepared substrate dissolved in acetate buffer at pH 6 and store at −20° until needed. Dilute this substrate solution with the same buffer to a concentration of 0.52 mg. of potassium hyaluronate per

[47] E. H. Kass and C. V. Seastone, *J. Exp. Med.* 79, 319-30 (1944); Albert Dorfman and Melvin L. Ott, *J. Biol. Chem.* 172, 367-75 (1948); G. H. Warren, J. G. Durso, and N. R. Levin, *Endocrinology* 43, 48-51 (1948); Roger L. Greif, *Proc. Soc. Exptl. Biol. Med.* 75, 813-15 (1950); *J. Biol. Chem.*, 194, 619-25 (1952); Harvey E. Alburn and Robert W. Whitley, *J. Biol. Chem.* 192, 379-93 (1951); Cf. Sibylle Tolksdorf, Marian H. McCready, D. Roy McCullagh, and Erwin Schwenk, *J. Lab. Clin. Med.* 34, 74-89 (1949); W. Escarón and H. Delfino, *Arch. soc. biol. Montevideo* 17, 41-50 (1950); H. Delfino and W. Escarón, *Arch. soc. biol. Montevideo* 18, [illegible] (1951); Viggo Faber, *Acta Pathol. Microbiol. Scand.* 32, 147-56 (1953).

[48] Roger L. Greif, *J. Biol. Chem.*, 205, 381-90 (1954); Sanford O. Byers, Alfred A. Tytell, and Milan A. Logan, *Arch. Biochem.*, 22, 66-86 (1949); Sibylle Tolksdorf, James W. Cassidy, Marion H. McCready, and D. Roy McCullagh, *Ann. N. Y. Acad. Sci.*, 52, 1024-7 (1950).

ml., and warm to 37.5°. Add 0.5 ml. of substrate at 20-second intervals, with mixing to successive tubes containing enzyme. Start a clock upon first contact of enzyme with substrate. During the incubation period, place standards containing 0.26, 0.208, 0.156, and 0.104 mg. of substrate, and blanks containing buffer alone, and enzyme alone, in tubes at 37.5°.

Procedure—*Macro.* Dilute plasma in acid-citrate-dextrose solution such as that discarded from a blood bank—1:10 with a citrate-phosphate buffer for pH 2.5 (Vol. I, page 176), containing 5.7 moles per liter of urea. Dilute 3 ml. of a 5 per cent aqueous solution of bromosulfalein [49] to 200 ml. with citrate-phosphate buffer for pH 2.5. Just before needed, mix equal parts of the diluted plasma and bromosulfalein solutions for use as color reagent.

After 30 minutes of incubation, add 2.5 ml. of warmed color reagent at 20-second intervals to the tubes, in the same order in which the substrate was added. Remove each tube from the bath immediately after addition of color reagent, mix, and let stand for 15 minutes at room temperature. Centrifuge, decant, and let drain. The size of precipitate is roughly proportional to the amount of unchanged substrate. Remove excess color reagent from the mouths of the tubes and add 2 ml. of 5 per cent sodium hydroxide solution to each. After solution is complete, dilute the contents of each tube to 10 ml. with water, mix, and read at 535 mμ.

Prepare a curve of nitrogen content of enzyme against optical-density readings. Draw a line to represent the optical density obtained with 0.13 mg. of substrate. The point corresponding to the intersection of the two lines represents the amount of enzyme, expressed as nitrogen, required to reduce the initial substrate concentration to one-half.

Micro. Modify the macro method as follows. To each of four 0.25 ml. portions of warmed enzyme solution, add 0.25 ml. of warmed substrate solution containing 0.02 mg. of potassium hyaluronate at intervals of 20 seconds. Use standard prepared as above but containing 0.02, 0.015, 0.01, and 0.005 mg. of substrate, also enzyme and solvent blanks. Add 1 ml. of color reagent to each and complete as for the macro method but dissolve in 2 ml. of 5 per cent sodium hydroxide solution and read at 570 mμ.

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[49] Supplied by Hynson, Westcott and Dunning, Inc.

Ketokinase

Ketokinase catalyzes the reaction of adinosine triphosphate with glucose to form glucose-6-phosphate. This acting as an acid is read by its effect on phenol red indicator at 588 mμ.[50]

Lipase

Lipase in the presence of sodium taurocholate gives the reaction of esterase in hydrolysis of β-naphthyl laurate to liberate β-naphthol.[51] Another substrate for this enzyme is butyrylsalicylic acid, the salicylic acid produced being read at 290-300 mμ.[52]

Procedure—To a series of tubes add the following: (1) 0.2 ml. of serum in 2 ml. of water; (2) 0.2 ml. of serum in 2 ml. of 4.3 per cent sodium taurocholate; and (3) as control 2 ml. of water. As buffer for pH 7.4 mix 58 ml. of 0.206 per cent sodium diethylbarbiturate with 42 ml. of 1:116 hydrochloric acid. As substrate mix 10 ml. of 0.2 per cent β-naphthyl laurate in acetone with 20 ml. of the buffer. Add 5 ml. of substrate to each tube and incubate for 5 hours at 37°. At this point the measure of activity is the β-naphthol content. As development reagent add 0.04 gram of dry tetrazotized di-*o*-anisidine to 10 ml. of water. After 2 minutes add 1 ml. of 40 per cent acetic acid. Add 1 ml. of this reagent to each tube and extract the purple color at once with 10 ml. of ethyl acetate. Centrifuge and read at 540 mμ against the control. Read in terms of β-naphthol.

The tubes without sodium taurocholate give the value for esterase. Those with sodium taurocholate give an increased color due to lipase. Interpret the results in terms of the definition: 1 unit of lipase in the presence of sodium taurocholate will liberate 0.01 gram of β-naphthol from β-naphthyl laurate in 5 hours at 37° in excess of that similarly liberated by esterase in the absence of sodium taurocholate. So tube (2) less tube (1) in terms of β-naphthol gives the value.

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[50] Jacques Wajzer, *Compt. rend.* **229**, 1270-2 (1949).

[51] Arnold M. Seligman, Marvin M. Nachlas, and Marie C. Mollomo, *Am. J. Physiol.* **159**, 337-42 (1949); Arnold M. Seligman and Marvin M. Nachlas, *J. Clin. Invest.* **29**, 31-6 (1950); Arnold M. Seligman and Philip Glotzer, *Surg. Forum, Proc. 36th Clin. Congr. Am. Coll. Surgeons*, **1950**, 478.

[52] B. H. J. Hofstee, *Science* **114**, 128-30 (1951).

Lipoid Oxidase

To estimate lipoid oxidase use it to form peroxides with linoleic acid. Then oxidize ferrous ion to ferric ion [53] and determine by the conventional thiocyanate reaction. While subject to direct reading, the color is also extractable with amyl alcohol. A unit of lipoid oxidase is defined as that which in the presence of 5 mg. of linoleic acid catalyzes the reaction of 0.001 mg. of oxygen in 1 minute at 25° and pH 7.

Procedure—As a buffer for pH 6.5 mix 55 ml. of a 2.1 per cent solution of citric acid with 45 ml. of 0.4 per cent sodium hydroxide solution and dilute to 1 liter. Mix 5 ml. of 0.1 per cent linoleic acid solution in acetone with 100 ml. of water and 5 ml. of the buffer solution. Adjust to 25°, add 1 ml. of the sample solution, and mix. After the desired reaction time add 10 ml. of concentrated hydrochloric acid to stop the reaction and mix. Add 1 ml. of 5 per cent ferrous ammonium sulfate in 1:10 hydrochloric acid and determine the peroxide present which will have oxidized the ferrous ion to ferric ion. To 10 ml. of the mixture exactly 15 minutes after addition of the ferrous ammonium sulfate add 10 ml. of ethanol to remove the turbidity due to the fat. Add 1 ml. of 20 per cent ammonium thiocyanate solution and read at 490 mμ. Subtract a blank in which the enzyme sample was replaced by water. Plot the curve from use of various time intervals and calculate from a time interval on the straight-line portion of the curve.

Lysozyme

Lysozyme, a mucolytic enzyme, is estimated by its ability to clear a standard susupension of the nonpathogenic organism *Micrococcus lysodeikticus*.[54] Colored extracts must be decolorized before turbidity readings are made. Results are accurate to about ±3 per cent.

Sample—*Gastic washings, feces.* Collect a sample in a refrigerated container and store at −18 to −23° F. The enzyme is stable for 3-4 months at this temperature. For analysis, partially thaw, weigh, and homogenize in a blender. To 10 grams of homogenate add 100 ml. of 1:150 hydrochloric acid, stir, and homogenize again. Freeze quickly in a deep freeze, thaw, and filter or centrifuge. Refreeze and thaw the

[53] R. J. Sumner, *Ind. Eng. Chem., Anal. Ed.* **15**, 14-15 (1943).

[54] Benjamin H. Pringle, Dario e. De Paulis, and Thomas D. Pemrick, *Am. J. Clin. Path.* **21**, 1039-44 (1951).

filtrate or supernatant fluid and pass through a sterile Seitz filter to remove bacteria.

Shake 10 ml. of the filtrate with 1-2 grams of 80-200 mesh alumina for about 15 minutes to remove color, centrifuge, and decant. Add 0.15 ml. of 4 per cent sodium hydroxide solution.

Prepare the culture to be used as substrate by growing Meyer's strain of *Micrococcus lysodeikticus* on tryptose-glucose-agar extract in quart milk bottles. Remove a 24-hour culture from the surface by adding 25 ml. of citrate-phosphate buffer for pH 6.2 (Vol. I, page 176). Decant the agar-free suspension into a 500-ml. flask and dilute with the citrate-phosphate buffer until the turbidity matches that of a McFarland No. 10 barium sulfate standard, whose transmittance has been previously established at 54 mμ.

Procedure—Pipet the extract, the buffer, and the bacterial suspension into tubes as follows:

Tube	*Buffer (ml.)*	*Original Extract (ml.)*	*Bacterial Suspension (ml.)*
1	1.0	2.0	3.0
2	2.0	1.0	3.0
3	2.5	0.5	3.0
4	2.7	0.3	3.0
5	2.8	0.2	3.0
6	2.9	0.1	3.0
7 (control)	3.0	0.0	3.0

Invert the tubes and warm at 37° for 1 hour. Add 1-2 drops of 4 per cent sodium hydroxide solution to each tube, mix, and transfer for turbidity measurement. A reading of 90 per cent transmittance ±3 per cent at 54 mμ is taken as complete clearing. One unit of lysozyme is the least amount of enzyme which results in complete clearing of the suspension.

Oxidase

α-Naphthol and dimethyl p-phenylenediamine hydrochloride are oxidized in alkaline solution to the blue indophenol.[55] Oxygen from the air causes this reaction to proceed slowly. The presence of oxidase accelerates it greatly. The difference between the color produced by the

[55] J. A. Dye, *Proc. Soc. Exptl. Biol. Med.* 24, 640-2 (1927); J. Laskowski, *Compt. rend. soc. biol.* 98, 1369-71 (1928); Michele Mitolo, *Boll. soc. ital. biol. sper.* 20, 829-31 (1945).

oxygen of the air and the color produced in the presence of oxidase affords a method for the determination of relative amounts of the latter.

The oxidation of *o*-phenylenediamine as catalyzed by oxidase gives a yellow to orange which is extractable with organic solvents. The optimum condition at 40° is pH 6.[56] Oxidation of *p*-cresol and catechol is a slightly less desirable alternative.

Sample—*Tissue.* Grind a sample of tissue with an equal weight of quartz sand in a mortar for 10 minutes.

Procedure—Prepare an 0.18 per cent solution of α-naphthol in a 1:1 water-ethanol, a 0.216 per cent aqueous solution of dimethyl *p*-phenylenediamine hydrochloride, and a 0.31 per cent aqueous solution of sodium carbonate. Mix 2 ml. of the α-naphthol solution with 2 ml. of the fresh diamine hydrochloride solution and 1 ml. of the sodium carbonate solution.

Transfer 1 gram of sand-sample mixture to a dish. Add 5 ml. of reagent, weight the dish and contents, and let stand for 10 minutes, stirring occasionally. Prepare a blank of 5 ml. of reagent at the same time. Add 19 per cent ethanol, if necessary, to bring the weight of sample and dish up to the previous value and the volume of the blank up to 5 ml. Add 10 ml. of ethanol. This makes the ethanol content about 70 per cent by volume. If the color is very dark, dilute with 70 per cent ethanol. Filter after 15 minutes. Read both against a sample blank.

Papain

A unit of papain is that amount which will liberate all the tyrosine from 1 mg. of egg albumin in 10 minutes at 36°. The liberated phenol is conveniently developed with phosphotungstic-phosphomolybdic acid.[57] The same method is applicable to denatured hemoglobin as substrate.[58]

Procedure—As a buffer for pH 7.4, mix 50 ml. of 6.8 per cent monopotassium phosphate solution with 39.5 ml. of 2 per cent sodium hydroxide solution and dilute to 200 ml. with water. Treat low-activity enzymes with sodium cyanide before the determination. Isolate the

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56 James S. Wallerstein, Ralph T. Alba, and Mary G. Hale, *Biochem. et Biophys. Acta* **1**, 175-83, 197-207 (1947).

57 José Erdos, *Enzymologia*, **14**, 8-9 (1950).

58 M. L. Anson, *J. Gen. Physiol.* **20**, 561-3 (1937).

standard substrate at about 55° from chicken egg white and dry *in vacuo*.

To 1 ml. of 0.10 per cent solution of ovalbumin and 5 ml. of buffer for pH 7.4 (Vol. I, page 176), add a measured amount of dispersed or solid sample. Shake vigorously for 30 seconds and place for 10 minutes in a water bath at 36°. Add 10 ml. of 0.3 per cent trichloroacetic acid solution to stop the reaction. Filter or centrifuge. Complete as for tyrosine by phosphomolybdic-phosphotungstic acid (page 126).

Pepsin

When pepsin acts on a standardized, homogenized suspension of coagulated egg white, the decrease of turbidity with time is a measure of pepsin present.[59] Chilling of the reaction mixture will completely retard peptic action for at least 0.5 hour and thus allow for readings.[60] Alternatively a 5.6 per cent solution of plasma at pH 2.1 is used and the color from phenol developed with phosphotungstic-phosphomolybdic acid reagent.[61]

By adding an unknown concentration of pepsin solution to dyed fibrin, the rate of digestion of the fibrin by the enzyme is estimated by the color liberated in the solution and used as a relative measure of the pepsin content. Various dyes used include cochineal,[62] alcohol blue, and Congo red.[63] Carbon monoxide-hemoglobin is a suitable substrate for pepsin estimation. The undigested hemoglobin is precipitated by trichloroacetic acid and the phenolic properties of the residue used for estimation of peptic activity.[64]

One unit of activity is that which will form, in 1 minute at 37° in 6 ml. of digestion mixture, an amount of hydrolyzed protein corresponding to 1 milliequivalent of tyrosine. If a dilute serum protein solution is

[59] Benjamin C. Riggs and William C. Stadie, *J. Biol. Chem.* **150**, 463-70 (1943).

[60] H. J. Wesselman and W. W. Hilty, *J. Am. Pharm. Assoc., Sci. Ed.* **37**, 357-9 (1948).

[61] J. N. Hunt, *Biochem. J.* **42**, 104-9 (1948).

[62] P. Grutzner, *Arch. ges. Physiol. (Pflugers)* **8**, 452-9 (1874); *Ibid.* **144**, 545-54 (1912); *Munch. med. Wochschr.* **58**, 768-9 (1911); W. Waldschmidt, *Arch. ges. Physiol. (Pflügers)* **143**, 189-229 (1911).

[63] I. A. Smorodinzew and A. N. Adowa, *Biochem. Z.* **153**, 14-18 (1924); *Z. physiol. Chem.* **149**, 173 (1925).

[64] M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.* **16**, 59-63 (1932); J. M. Beazell, C. R. Schmidt, A. C. Ivy, and J. F. Monoghan, *Am. J. Digestive Diseases Nutrition* **5**, 661-3 (1938).

acted on by a pepsin solution for a definite time at a controlled pH value, the amount of undecomposed protein is estimated nephelometrically by thiosalicylic acid.[65]

Sample—*Gastric juice.* Centrifuge the specimen for at least 5 minutes and use the middle layer.

Procedure—*Turbidimetric.* To prepare the egg white substrate, boil a fresh hen's egg for 10 minutes. Wash free of membrane, remove the yolk, and wash the coagulated egg white in a mortar. While adding 5 ml. of water per gram, pass through a homogenizer several times. Double the volume with water, homogenize again, and centrifuge for 15 minutes. Remove any surface film and analyze the supernatant fluid for total nitrogen. Dilute to contain 0.5 mg. of nitrogen per ml. Add merthiolate to give a concentration of 1:10,000. This substrate can be stored for 2 months at below 10°.

Mix 1 ml. of centrifuged specimen and 4 ml. of buffer solution consisting of a 4:1 mixture of 1:60 hydrochloric acid and 1.9 per cent sodium citrate solution. Bring to 30° ±0.1° in a water bath and add 5 ml. of substrate previously heated to the same temperature. Mix within 8-10 seconds. At 30 seconds place in the photometer and at exactly 1 minute make a reading at 440 mμ. Replace in the water bath, remove at 5.5 minutes, and make a reading at 6 minutes. Measure time to within 2 seconds.

By dyed fibrin. The reagent is specially prepared. Grind fresh fibrin, wash free of all blood coloring, and keep in glycerol. Cut the fibrin in small pieces with scissors and wash free of glycerol. Press out the water and introduce into a 0.05 per cent solution of alcohol-blue in glycerol, using 1 part of moist fibrin to 4 parts of dye solution. Let stand for 24 hours, stirring occasionally. The fibrin is stained a dark blue-violet and keeps indefinitely in the dye solution. To a 0.3-gram portion of dyed fibrin add 12.5 ml. of sample and 2.5 ml. of 1:350 hydrochloric acid. Read after a suitable period for digestion.

By carbon monoxide-hemoglobin. As reagent, bubble carbon monoxide through whipped ox blood. Centrifuge and siphon off the serum and white cells. Wash the hemoglobin 4 times with cold 0.9 per cent sodium chloride solution. Add an equal volume of water and one-sixth volume of toluene. Bubble carbon monoxide through the solution and shake

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[65] P. Rona and Hans Kleinmann, *Biochem. Z.* **155**, 34-53 (1925); *Ibid.* **159**, 146-74 (1925).

vigorously. Let stand overnight and siphon off the hemoglobin layer.

Prepare alumina cream by adding a slight excess of 1:25 ammonium hydroxide to a 1 per cent solution of ammonium alum at room temperature. Wash the precipitate by decantation until the wash waters show only a faint trace of residue on evaporation.

Mix the hemoglobin solution with one-tenth volume of the alumina cream. Centrifuge and filter. Dialyze overnight in a shaking dialyzer and store under carbon monoxide in the cold with toluene as the preservative. Estimate the concentration of the hemoglobin solution by the Kjeldahl method, taking the nitrogen content of hemoglobin as 17.7 per cent. Dilute to 5 per cent. Mix 2 volumes of this solution with 3 volumes of 1:120 hydrochloric acid. When stored for 10 days, or when the concentration is either 3 per cent or 1.5 per cent instead of 2 per cent, there is no significant effect on the rate of digestion.

Adjust 5 ml. of the carbon monoxide-hemoglobin solution to 37° or 25° and add 1 ml. of enzyme solution in 4 seconds. The middle of the addition is the start of the time. Mix. After exactly 5 minutes add 10 ml. of 4 per cent trichloroacetic acid solution and mix well. Measure out 3 ml. of the filtrate, add 20 ml. of water, 1 ml. of 14.6 per cent sodium hydroxide solution and 1 ml. of phosphotungstic-phosphomolybdic acid reagent (Vol. III, page 116). Read at the end of 10 minutes against a reagent blank.

Peroxidase

Oxidation of leuco-2,6-dichlorobenzenoneindo-3′-chlorophenol is catalyzed by peroxidase in the presence of hydrogen peroxide and the rate of color formation is proportional to enzyme concentration.[66] A pH of 6 is necessary for this reaction. Phenol oxidase and cytochrome oxidase could interfere, but do not due to high dilution. This is a more sensitive reaction than that with pyrogallol. If dilute 2,6-dichlorophenol-indophenol is titrated with ascorbic acid, the titrated mixture gives a blue pigment with peroxidase.[67] The blue pigment is extracted with ether and the resulting red solution is read.

Peroxidase will oxidize pyrogallic acid to gallopurpurin in the presence of hydrogen peroxide and the resulting color is used for esti-

[66] Frederick G. Smith and Elmer Stotz, *J. Biol. Chem.* 179, 865-80 (1949); Frederick G. Smith, Willard B. Robinson, and Elmer Stotz, *Ibid.* 179, 881-9 (1949).

[67] W. Diemair and H. Häusser, *Z. anal. Chem.* 122, 12-14 (1941).

mation of the peroxidase content.[68] If polyphenoloxidase and catalase are present, the activity of the former is eliminated by removal of atmospheric oxygen and the latter is inhibited by sodium nitrate.[69] Similar applicable reactions are those with guaiacol and hydrogen peroxide,[70] or with benzidine and hydrogen peroxide.[71] Another alternative is *o*-phenylenediamine and hydrogen peroxide,[72] of which the color is extractable with butanol. The conversion of phenolphthalein to phenolphthalin is another measure of peroxidase activity.[73] The end product is read in alkaline solution.

Sample—*Plant tissue.* Grind a fresh or frozen weighed sample in a blender to rupture the cells. Add sufficient 3.5 per cent dibasic potassium phosphate solution to maintain a pH of 7-8. Dilute with 0.5 per cent gelatin solution so that the dilution of tissue in homogenate is 1:10, and assay immediately by leuco-2,6-dichlorobenzenoneindo-3′-chlorophenol.

Blood. Dilute 1:400 for development by pyrogallic acid, 1:5 for development by benzidine, or 1:1000 for development by guaiacol and hydrogen peroxide.

Dried blood. Pulverize the sample and place in a desiccator. Weigh about 1 mg. of sample and dissolve in 10 ml. of buffer for pH 5 (Vol. I. page 176). Peroxidase dissolves quickly from the powdered blood. Let stand for 1 hour and filter. In general the peroxidase content decreases with the age of the blood stains. Develop by guaiacol and hydrogen peroxide.

Procedure—*By leuco-2,6-dichlorobenzenoneindo-3′-chlorophenol.* To 1 ml. of sample solution add 2.5 ml. of 0.2 *M* phosphate buffer for pH 6 (Vol. I, page 176), 7.5 ml. of 0.02 per cent solution of the leuco dye, and 1 ml. of 0.48 per cent hydrogen peroxide. Mix by swirling and read at 645 mμ against a reagent blank.

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68 Jorgen Bielefeldt, *Z. Vitaminforsch.* **13**, 286-94 (1943); Jorma Erkama, *Suomen Kemistilehti* **19B**, 32-4 (1946); Cf. E. Nicodemi, *Atti soc. lombarda sci. med. biol.* **3**, 90-2 (1948).

69 M. A. Bokuchava, *Doklady Akad. Nauk SSSR* **60**, 409-12 (1948).

70 A. Bach and Sophie Zubkowa, *Biochem. Z.* **125**, 283-91 (1921); Fritz Schwartz, *Deut. Z. ges. Gericht. Med.* **27**, 1-34 (1936).

71 Giuseppe Scoz and Maria Giulia Ronzoni, *Ann. ist. Carlo Forlanini* **12**, 356-60 (1946).

72 James S. Wallerstein, R. T. Alva, M. G. Hale, and H. Levy, *Biochem. et Biophys. Acta* **1**, 327-34 (1947).

73 Morizo Ishidate and Sadasuke Okano, *J. Pharm. Soc. Japan* **63**, 220-8 (1943).

By 2,6-dichlorophenol-indophenol and ascorbic acid. Titrate 0.03 per cent 2,6-dichlorophenol-indophenol solution with 0.05 per cent ascorbic acid. Mix 1 ml. of sample, 1 ml. of citrate buffer for pH 6, 1 ml. of 6 per cent hydrogen peroxide, and 1 ml. of reagent. Extract the blue with ether and read the residual red against a sample blank.

By pyrogallol and hydrogen peroxide. Polyphenoloxidase and catalase absent. Mix 5 ml. of fresh 0.5 per cent solution of pyrogallol, 0.5 ml. of a 1:90 dilution of 30 per cent hydrogen peroxide, and 5 ml. of 1:5 dilution of 0.2 *M* acetate buffer for pH 6 and dilute to about 20 ml. Prepare a sample blank at the same time, omitting the pyrogallol. Add 1 ml. of sample to each and dilute each to 25 ml. Stir each promptly upon addition of the enzyme. Measure the time from the mixing of the sample until the value of $E_5 = 1.01$ at 470 mμ. Then peroxidase content = 0.059/(t in minutes × grams of enzyme preparation).

Polyphenoloxidase and catalase present. The apparatus consists of a test tube having a ground-glass joint side-arm connected to a swivelled bulb. Make a similar attachment to the constricted upper opening of the test tube and then connect a pump at the top of the vessel. To 1 ml. of sample add 0.1 ml. of 1 per cent sodium nitrate solution, 3 ml. of buffer for pH 5.3 (Vol. I, page 176) and 5 ml. of water. Charge the lower movable bulb with 1 ml. of 1 per cent pyrogallol or pyrocatechol solution and 2 ml. of 1 per cent hydrogen peroxide. Charge the upper bulb with 3 ml. of 20 per cent sulfuric acid to stop the reaction at the end of the run. Evacuate to 8-10 mm. in 2-3 minutes and disconnect the pump. Discharge the contents of the lower bulb into the vessel and incubate at the desired temperature. At the end of the run, discharge the upper bulb into the reaction vessel, release the vacuum, and read the absorption.

By benzidine and hydrogen peroxide. Blood. Mix 18 ml. of water, 2 ml. of 0.82 per cent anhydrous sodium acetate solution containing 1 per cent of benzidine, and 2 ml. of 1:8 dilution of 30 per cent hydrogen peroxide. When the solution is at the temperature of the ice bath, add 1 ml. of 1:5 blood which will represent 1 per cent of the mixture. Read after 5 minutes.

By guaiacol and hydrogen peroxide. Mix 3 ml. of the sample solution, 1 ml. of 0.1 per cent guaiacol solution, and 1 ml. of 1 per cent hydrogen peroxide solution. Let this stand for 30 minutes and read against a reagent blank.

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67 V.

Phenol Oxidase

A phenol in the presence of oxygen and an oxidase forms a quinone. The latter in turn will react with a leuco dye to give the phenol and the dye.[74] Reading the color intensity of the dye then measures the amount of phenol oxidase present. An appropriate dye is 2-6-dichlorobenzeneoneindo-3′-chlorophenol. While any phenol may be used, catechol is appropriate.

Sample—*Tissue.* Disintegrate a weighed sample of fresh or frozen tissue with ten parts of water in a blender until the cells are ruptured, the water containing sufficient 3.5 per cent dipotassium phosphate solution to maintain the pH at about 7. Dilute to an appropriate concentration for assaying. Refrigerate if delay before determination is unavoidable.

Procedure—As buffer mix 12.6 ml. of 2.84 per cent solution of anhydrous disodium phosphate with 7.4 ml. of 1.92 per cent citric acid solution to give a pH of 6. As a stock solution of 2,6-dichlorobenzeneoneindo-3′-chlorophenol dissolve 35 mg. per 100 ml. of water by shaking intermittently for 30 minutes and filtering. This is stable for 3-4 days. For use dilute 40 ml. to 200 ml. and add 1 ml. of fresh 0.2 per cent suspension of 5 per cent palladized asbestos. Pass hydrogen through until the dye is just reduced. Normally this occurs within 15 minutes. Add 4 ml. of the buffer and filter on a Büchner funnel. Pass a slow stream of hydrogen through the dye solution until it is used. Dry the washed palladized asbestos for reuse. A red dye solution indicates that either too much asbestos was used or hydrogen was passed through after reduction was complete.

With all reagents at 30°, mix 5 ml. of buffer, 5 ml. of the reduced dye solution, 1 ml. of 1.1 per cent catechol solution, and 1 ml. of the sample extract. Read at 5-second intervals over the first 45-60 seconds, using 645 mμ. Plotted on semilog paper, the slope of the straight portion of the line gives the rate and therefore the enzyme activity of the sample.

Phosphatase

Serum contains relatively large amounts of alkaline-active phosphatase with very small amounts of an acid-active form.[75] An approp-

[74] Frederick G. Smith and Elmer Stotz, *J. Biol. Chem.* **179**, 865-79 (1949).

[75] Ethel Benedict Gutman and Alexander B. Gutman, *Ibid.* **136**, 201-9 (1940).

riate substrate for determining the alkaline form is *p*-nitrophenyl phosphate, which is colorless. Upon splitting off of the phosphate group, the yellow salt of *p*-nitrophenol is liberated; hence the hydrolyzed substrate acts as an indicator of the amount of splitting and thus a measure of phosphatase activity.[76] The method [77] consists merely of incubating the serum with the buffered reagent, stopping the reaction by diluting with alkali, and measuring the amount of color developed. Serum itself contributes little to the color, and, by the addition of acid after reading, the yellow sodium salt is converted to colorless free nitrophenol for reading as a sample blank. The method is also applicable to acid phosphatase.

Disodium β-glycerophosphate is hydrolyzed by phosphatase and the liberated phosphate estimated as a measure of activity. By using veronal as a buffer for pH 8.6 the period of incubation is reduced 1 hour.[78] The activity is defined as the number of mg. of phosphorus liberated from sodium β-glycerophosphate substrate as the phosphate ion during 1 hour, at pH 8.6 and 37°, when hydrolysis does not exceed 10 per cent of the substrate. As the products of hydrolysis do not cause an appreciable error up to 10-12 per cent, the presence of excess glycerophosphate interferes with some methods of estimation of phosphate, but a molybdate method is satisfactory. When sera are high in phosphatase, the calculated maximum error is 3-4 per cent.

The use of disodium phenylphosphate offers advantages such as convenience of estimation of phenol and at least twice the rapidity of hydrolysis.[79] Results are expressed as mg. of phenol liberated by hydrolysis by 100 ml. of serum from excess disodium phenylphosphate, at pH 9 for 30 minutes at 37°, each mg. of phenol liberated being referred to as a unit of phosphatase. Within the range of 10-100 units

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[76] Earl J. King and George E. Delory, *Biochem. J.* **33**, 1185-90 (1939); Otto A. Bessey, Oliver H. Lowry, and Mary Jane Brock, *J. Biol. Chem.* **164**, 321-9 (1946).

[77] Marie A. Andersch and Adam Szczypinski, *Am. J. Clin. Path.* **17**, 571-4 (1947).

[78] L. Michaelis, *J. Biol. Chem.* **87**, 33-5 (1930); H. D. Kay, *Ibid.* **89**, 235-47 (1930); Aaron Bodansky, L. Hallman and R. Bonoff, *Proc. Soc. Exp. Biol. Med.* **28**, 762-3 (1931); Aaron Bodansky, *J. Biol. Chem.* **101**, 93-104 (1933); Otto Hövels, *Z. Kinderheilk.* **66**, 237-45 (1949).

[79] Earl J. King and A. Riley Armstrong, *Can. Med. Assoc. J.* **31**, 376-81 (1934); Alejandro Martín, *Rev. soc. argentina biol.* **20**, 636-43 (1944); Péter Bálint, István Komáromy and Marianne Lenner, *Orvosok Lapja Népegészségügy* **3**, 1233-4 (1947); Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, 7th Ed., pages 240-5, The Association of Official Agricultural Chemists, Washington, D. C. (1950); Cf. Fred Ruppert, *Z. ges. exptl. Med.* **116**, 378-83 (1950).

the error is 1-2 per cent. Suitable methods of development are by phosphotungstic-phosphomolybdic acid or by 2,6-dibromoquinone chlorimide.

Another method involves the use of a soluble phenolphthalin phosphate which is water clear.[80] Enzymic decomposition liberates phenolphthalein for reading in alkaline solution. The amount of enzyme which will liberate the colorimetric equivalent of 1 mg. of phenolphthalein from excess substrate in 1 hour at 37° under optimum conditions of pH is defined as 10 units of acid or alkaline phosphatase. Thus the hydrolysis is slow with this substrate. The curve does not conform to Beer's law. Another substrate is *o*-carboxyphenylphosphoric acid prepared by reaction of salicylic acid and phosphorus pentachloride.[81]

Sample—*Serum.* As substrate for the sodium glycerophosphate method, dissolve 2.5 grams of sodium glycerophosphate and 2.12 grams of monosodium diethylbarbiturate in carbon dioxide-free water and dilute to 500 ml. Keep in an ice box in bottles covered with a 3-cm. layer of washed petroleum ether.

Measure 10 ml. of substrate into a tube, avoiding introduction of air. Place in a water bath at 37° for 5-10 minutes. Add 1 ml. of serum from which suspended matter has been centrifuged. Mix by inversion once and heat in the bath at 37° for exactly 1 hour. Remove, cool at once in ice water, and add 9 ml. of 10 per cent trichloroacetic acid solution. Mix and filter through quantitative paper after a few minutes.

Mix 2 ml. of serum with 9 volumes of 5 per cent trichloroacetic acid solution and filter. Inorganic phosphorus as estimated on this filtrate is the correction for the determination made on the serum with substrate for the sodium glycerophosphate method.

Blood. Collect blood from a vein in the usual manner and prevent clotting by addition of a minimal amount of oxalate. Add 2 ml. to the substrate as described for serum and prepare a sample for inorganic phosphorus as there described.

Alternatively, let 5 ml. of blood, free from anticoagulant, stand in a tube until a clot has formed. Loosen this from the sides of the tube and centrifuge. Pour the serum into a fresh tube and centrifuge again to separate any residual suspended matter. The serum can be stored in

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[80] Charles Huggins and Paul Talalay, *J. Biol. Chem.* **159**, 399-410 (1945); Heinz Jancker and Willibald Diemair, *Z. anal. Chem.* **130**, 56-7 (1949).

[81] Hans Brandenberger and Roberta Hanson, *Helv. Chim. Acta.* **36**, 900-6 (1953).

a refrigerator for 24 hours without serious loss of accuracy. Determine by disodium phenylphosphate.

Bile. Dilute bile 1:10 and 1:100 with physiological saline and treat as for the serum from blood. The two dilutions are necessary since some biles contain as much as 7000 units per ml. Determine by disodium phenylphosphate.

Milk. Dilute the sample with an equal quantity of water. Determine by means of phenolphthalin phosphate substrate.

Urine. Use freshly voided samples and determine by means of phenolphthalin phosphate substrate.

Procedure—*By p-nitrophenyl phosphate as substrate.* Dissolve 7.5 grams of glycine and 95 mg. of magnesium chloride in 700-800 ml. of water. Add 85 ml. of 4 per cent sodium hydroxide solution and dilute to 1 liter. Prepare a 0.4 per cent solution of disodium *p*-nitrophenyl phosphate in 1:12,000 hydrochloric acid, and adjust the pH to 6.5-8. As reagent mix equal parts of the two preceding solutions and adjust to pH 10.3-10.4. Freeze or refrigerate for storage and, when 2 ml. plus 10 ml. of 0.08 per cent sodium hydroxide solution have an extinction greater than 0.1, discard or extract with water-saturated butanol and then with ether, and readjust the pH.

With 5 cmm. of serum. Place the sample in a tube and immerse in a shallow pan of ice water. Rapidly add 50 cmm. of ice-cold reagent and mix by tapping carefully. Transfer to a 38°-water bath at a depth sufficient to cover the bottom half of the tube. After exactly 30 minutes return to the pan of ice water and add 0.5 ml. of 0.08 per cent sodium hydroxide solution to stop the reaction. Read at 400 mμ, add 2-4 cmm. of concentrated hydrochloric acid, and make a second reading as a sample blank. Subtract the sample blank from the first reading.

With 0.1 ml. of serum. Use 1 ml. of reagent and 20 ml. of 0.08 per cent sodium hydroxide solution. Because of the large volumes, allow the reagent tube in the water bath to come to temperature and then add the serum. Exactly 30 minutes later add 20 ml. of 0.08 per cent sodium hydroxide solution and complete as before.

By glycerophosphate. Dilute a sample to 4 ml. with water. Dissolve 90 grams of ammonia-free molybdic acid in 250 ml. of 20 per cent sodium hydroxide solution and dilute with water to 2 liters. This solution should be faintly alkaline to phenolphthalein. Mix 1 volume of this with 1 volume of 1:2.5 sulfuric acid and 2 volumes of water. Add 4 ml. of this reagent to sample and standard. Mix and add 1 ml. of 60

per cent stannous chloride solution in concentrated hydrochloric acid, freshly diluted 1:200 with water. Mix and read in terms of a phosphoric acid standard. A reagent blank should be colorless or faintly blue or green.

By disodium phenylphosphate. Biological samples. As reagent mix equal parts of 0.218 per cent disodium phenyl phosphate solution and 4.2 per cent citric acid solution containing 376 ml. of 4 per cent sodium hydroxide solution per liter. Adjust the pH to 4.9. Transfer 10 ml. of reagent to a thermostat at 37°. Add 0.5 ml. of sample. Stopper, mix, and leave in the thermostat exactly 3 hours. Add 4.5 ml. of phosphotungstic-phosphomolybdic acid reagent (Vol. III, page 116), diluted 1:3 with water. Mix and filter. At the same time, as control mix 10 ml. of substrate with 0.5 ml. of sample and at once add 4.5 ml. of the diluted reagent. Mix and filter. Measure 10 ml. of filtrate from sample and control and add to each 2.5 ml. of 20 per cent sodium carbonate solution. Mix and place in a thermostat for 5 minutes to develop the blue color. Read at 720 mμ and interpret in terms of a phenol standard.

Food products. Milk. As a buffer substrate dissolve 1.09 gram of disodium phenyl phosphate and 11.54 grams of sodium diethyl barbiturate in water saturated with chloroform and dilute to 1 liter. Add 10 ml. of chloroform and refrigerate.

To 10 ml. of substrate add 0.5 ml. of sample and a few drops of chloroform. Mix by rotating the tube and cover but do not stopper. Warm to 37-39° and incubate more than 18 and less than 24 hours. Add 4.5 ml. of dilute phosphotungstic-phosphomolybdic acid reagent (Vol. III, page 116). Mix and after 3 minutes filter. To 5 ml. add 1 ml. of 14 per cent sodium carbonate solution and mix. Heat for 5 minutes in boiling water, filter, and read at 650 mμ. Interpret from a phenol curve similarly treated except that incubation is unnecessary.

To check for deterioration of the reagent and presence of interfering substances mix 10 ml. of substrate, 4.5 ml. of dilute reagent, and 0.5 ml. of sample. Without incubation complete from "Mix and, after 3 minutes, filter." Subtract 0.02 mg. from the phenol value so obtained and subtract that result from the determined value as a correction.

By sodium phenolphthalin phosphate. To prepare an alkaline-buffered substrate, mix 20.6 grams of sodium barbital and 0.608 gram of sodium phenolphthalin phosphate and dilute to 1 liter. Add 7.5 ml. of chloroform as preservative. As glycine buffer, add with stirring 100 grams of sodium hydroxide to 100 ml. of water in a beaker immersed

in water. Add 15 ml. of this solution to an aqueous solution of 9.19 grams of glycine and 7.17 grams of sodium chloride and then dilute nearly to 1 liter with water. Add 40 grams of powdered sodium pyrophosphate decahydrate and dilute to 1 liter. The pH is 11.2.

Add 0.5 ml. of sample solution to 5 ml. of buffered substrate which has been in a water bath at 37° for about 5 minutes. Mix by rotating, not by inversion. Incubate for exactly 1 hour and then add 4.5 ml. of the glycine buffer and read immediately at 480 mμ against a reagent blank.

By 2,6-dibromoquinone chlorimide. Milk. As a barium borate-hydroxide buffer for pH 10.6 ± 0.15 dissolve 25 grams of fresh barium hydroxide octahydrate in water and dilute to 500 ml. Separately dissolve 11 grams of boric acid in water and dilute to 500 ml. Warm the solutions to 50°, mix, cool to about 20°, filter, and stopper for storage. For use dilute with an equal volume of water. As a borate buffer for pH 9.8 ± 0.15 dissolve 6 grams of sodium metaborate and 20 grams of sodium chloride in water and dilute to 1 liter.

As a substrate for pasteurized milk dissolve 0.1 gram of phenol-free sodium phenylphosphate in 100 ml. of a 1:1 mixture of the barium borate-hydroxide buffer with water. For use with raw milk use 0.2 gram of the phosphate.

To a blank and sample add 1 ml. of milk sample. For goat's milk use 3 ml. of sample. Heat the blank in boiling water for about 1 minute to inactivate. For milk add 10 ml. of borate substrate to each tube. For old or slightly sour cream reduce this to 8 ml. and 2 ml. of water. This substrate is satisfactory for fresh milk, sweet buttermilk, and cheese whey. For old or slightly sour milk make the substrate with undiluted buffer. For chocolate drinks dilute the buffer with 1/4 volume of water. For buttermilk under pH 4.5 increase the barium hydroxide in the buffer to 26 grams. For goat's milk increase it to 27 grams. Adjust unknown samples to pH 10-10.05.

After adding the substrate, incubate at 37-38° for 1 hour, shaking occasionally. Heat in boiling water to 85-90°, which will take about 1 minute, and cool in cold water. Add a solution of 3 per cent zinc sulfate heptahydrate and 0.6 per cent copper sulfate pentahydrate as follows. For fresh milk, sweet buttermilk, cheese whey, or fresh cream use 1 ml. For old or slightly sour milk or acid buttermilk use 1 ml. of 6 per cent zinc heptahydrate solution. For chocolate drinks use 1 ml. of a solution containing 4.5 per cent of zinc heptahydrate and 0.1 per cent of copper

sulfate pentahydrate. For old or slightly sour cream use 1 ml. of 4.5 per cent solution of zinc sulfate heptahydrate. The final pH should be 9.0-9.1. Filter and to 5 ml. add 5 ml. of color development buffer. The pH should now be 9.3-9.4.

As 2,6-dibromoquinone chlorimide reagent dissolve 40 mg. in 10 ml. of absolute methanol or ethanol and refrigerate in the dark. Add 4 drops of this to each tube and mix. After 30 minutes read at 610 mμ and subtract the blank. Read in terms of a phenol curve. If the color is not sufficiently intense, extract with 5 ml. of butanol, centrifuge to break the emulsion, and read the butanol layer.[82]

Proteolytic enzyme activity

With azoalbumin as substrate, proteolytic activity is determined for any enzyme active above pH 5 or below pH 3, by means of the color of the trichloroacetic acid filtrate of the digested substrate.[83] Alternatively, determine by applying them to the nitrated protein, subsequently deproteinizing, and development of the color of the split nitrated products.[84]

Sample—*Gastric juice.* As buffer dissolve 42.016 grams of crystalline citric acid in 200 ml. of 8 per cent sodium hydroxide solution and dilute to 1 liter. Mix 9 ml. with 18 ml. of 1:60 hydrochloric acid and adjust to pH 1.6 by dropwise addition of 1:1 hydrochloric acid. Centrifuge the sample and remove 1 ml. from the middle layer. Dilute to 20 ml. with the buffer.

Duodenal juice. Centrifuge and remove 1 ml. from the middle layer. Dilute to 100 ml. with 0.5 N ammonium hydroxide-ammonium chloride buffer for pH 9.5 for use as sample.

Procedure—As substrate dissolve 50 grams of bovine plasma albumin in 1 liter of water and add, with stirring, 10 grams of sodium bicarbonate suspended in 100 ml. of water. Dissolve 0.025 mole of sulfanilic acid in 200 ml. of water containing 5 ml. of 20 per cent sodium hydroxide solution and add 0.1725 gram of sodium nitrite. Stir and add 10 ml. of 1:1 hydrochloric acid. Stir and add 10 ml. of 20 per cent sodium hydroxide solution. Stir a few seconds and add quickly to the alkaline

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82 The original reference provides for several other special cases.

83 Rudolph M. Tomarelli, Jesse Charney, and Mary Lord Harding, *J. Lab. Clin. Med.* **34**, 428-33 (1949).

84 E. V. Pechmann, *Biochem. Z.* 321, 248-60 (1950).

albumin solution. Stir and dialyze against cold tap water overnight. The dialyzed solution is then lyophilized. Use the orange dialyzed solution of azoalbumin as substrate.

To 1 ml. of substrate add 1 ml. of diluted enzyme sample and digest for 30 minutes at 38°. Remove undigested substrate from solution with 8 ml. of 5 per cent trichloroacetic acid. Filter. To a 5-ml. aliquot of filtrate add 5 ml. of 2 per cent sodium hydroxide solution and read against a similarly prepared blank containing no enzyme at 445 mμ. The activity of the digestive juice is expressed in terms of the velocity constant of the enzymatic reaction, $K = 1/t\ 2.3 \log C_1/C_2$, where C_1 and C_2 are initial and final protein concentrations respectively, after 4 minutes of digestion. Optical densities are substituted for concentrations after taking consideration of the aliquots.

Determine the initial concentration, C_1, by adding 5 ml. of 2 per cent sodium hydroxide solution to a 5-ml. aliquot of a 1:200 dilution of the substrate solution and read at 445 mμ. Multiply by 20 to give C_1. Determine the concentration after digestion, C_2, by subtracting the reading for the trichloroacetic acid filtrate from the C_1 value. Calculate the velocity constant for the diluted solution and multiply by the dilution factor to obtain the reaction constant for the undiluted juice.

Pseudocholinesterase

This form of cholinesterase is covered under that topic (page 499).

Succinic dehydrogenase

Methods applied to enzymes which reduce cytochromes are applicable to succinic dehydrogenase.[85] Thus cytochrome C is the color reagent. An alternative is reduction of tetrazolium chloride to a red insoluble formazan,[86] or reduction of 2,6-dichloroindophenol.[87]

Succinic dehydrogenase activity of plant tissues is determined by its anaerobic reduction of the tetrazolium salt, p,p′-diphenylenebis-2-(3,5-diphenyltetrazolium chloride).[88] The color is read in an organic solvent. The method is not specific but can be applied to the study of multiple

[85] S. J. Cooperstein, Arnold Lazarow, and Nancy J. Kurfess, *J. Biol. Chem.* **186**, 129-39 (1950).

[86] Ernest Kun and L. G. Abood, *Science* **109**, 144-6 (1949).

[87] Otomar Pihar, *Chem. Listy*, **46**, 379-82 (1952).

[88] Eugene Glock and Clifford O. Jensen, *J. Biol. Chem.* **201**, 271-8 (1953).

enzyme systems. Malonate inhibits up to 70 per cent of succinic dehydrogenase.

Samples—*Tissue.* Homogenize with 5 volumes of ice-cold buffer for pH 7.4, diluted to M/30 (Vol. I, page 176). Dilute with the same buffer as needed and develop by cytochrome C. For development by 2,6-dichloroindophenol similarly homogenize but with ice water.

Seeds and seedlings. Soak for 2 hours in water and spread on moist cheesecloth in glass-covered trays. Let seeds germinate in the dark at 20°, the time depending on the phase of the investigation. Mince the tissues *in vacuo* in a blender for 3 minutes with twice their weight of cold 0.1 *M* potassium phosphate buffer at pH 7.4 (Vol. I, page 176). Remove cellular debris by squeezing the blended material through cheesecloth. Centrifuge the liquid. Decant the supernatant fluid and use immediately for development by tetrazolium salt.

Procedure—*By cytochrome C.* Mix 0.5 ml. of 0.14 per cent cytochrome C in 0.17 *M* phosphate buffer for pH 7.4 (Vol. I, page 176), 2 ml. of water, and 0.3 ml. of an aqueous solution containing 0.1 per cent of aluminum chloride hexahydrate and 0.045 per cent of anhydrous calcium chloride.

Mix 0.1 ml. of 8.1 per cent sodium succinate solution in the phosphate buffer for pH 7.4 with 0.02 or 0.04 ml. of sample solution. After 2 minutes add 0.1 ml. of 0.15 per cent sodium cyanide in the phosphate buffer for pH 7.4. Add 2.8 ml. of the cytochrome C solution. Read at 550 mμ every 30 seconds for 3 minutes. Then reduce the cytochrome C by adding a few crystals of sodium hydrosulfite and read as the original color from which is to be subtracted the earlier readings. Plotting the logarithm of the difference against time is a measure of the enzyme activity.

By 2,6-dichloroindophenol. As buffer mix 17.805 grams of disodium phosphate dihydrate in water with 20 ml. of *N* hydrochloric acid and dilute to 1 liter. Mix 0.05 ml. of sample, 1 ml. of the buffer, 0.1 ml. of 0.0147 per cent sodium cyanide solution, 0.45 ml. of water, and 0.2 ml. of 0.015 per cent solution of 2,6-dichloroindophenol. After 3 minutes add 2 ml. of 8.1 per cent solution of sodium succinate. Read at 610 mμ after 15 seconds and after 30 seconds.

By tetrazolium salt. To a Thunberg tube add 1 ml. of freshly prepared enzyme extract, 0.5 ml. of either 0.2 *M* substrate or water, 1 ml.

of 0.1 *M* phosphate buffer at pH 7.4 (Vol. I, page 176), and 2 ml. of water. In the bulbed side-arm stopper place 0.5 ml. of 2 per cent tetrazolium-salt solution. For blanks, heat a portion of enzyme solution to inactivate the enzyme and treat in the same way. Evacuate the Thunberg tubes for 3 minutes at 10-15° and place in a water bath at 37° for 10 minutes. Tip the tetrazolium-salt solution from the side-arm into the main part of the Thunberg tube and let react at 37° for 4 hours. Stop the reaction by mixing with 10 ml. of water-saturated *n*-butanol. Centrifuge and read the clear supernatant butanol extract at 520 mμ. Report in terms of micrograms of tetrazolium salt reduced per gram of fresh tissue.

Tannase

The enzyme action of tannase solutions is measured by estimating the rate at which gallotannins are hydrolyzed to gallic acid.[89]

Procedure—Prepare the necessary solution of the enzyme tannase by shaking 1 gram of mycelium powder with 50 ml. of water for 3 hours. Let this stand for 21 hours and filter. To 10 ml. of a gallotannin solution containing 3 grams per liter add 1 ml. of the enzyme solution to be standardized. Prepare a number of these samples, according to the number of determinations to be made. Pour a layer of benzene on the surface of each sample solution, stopper tightly, and place in an incubator at 25°.

Determine the gallic acid in one of the sample solutions at the end of each 24-hour period. To the sample for analysis add 4.5 ml. of a 1 per cent solution of quinine hydrochloride and 0.5 ml. of a 16 per cent solution of sodium chloride to coagulate the gallotannins. Filter and determine by the method for gallic acid, using ferrous sulfate and sodium potassium tartrate (Vol. III, page 411).

Transaminase

Transaminase activity in tissues is determined indirectly by the action of this enzyme in converting aspartic acid to oxalacetic acid. The latter is converted chemically to pyruvate, which is determined by reaction with dinitrophenylhydrazine.[90] The method is accurate to about

[89] Hans Kleinmann and G. Scharr, *Biochem. Z.* **252**, 145-84 (1932); B. J. Krijgsman, *Z. physiol. Chem.* **228**, 256-67 (1934).

[90] N. E. Tonhazy, N. G. White, and W. W. Umbreit, *Arch. Biochem.* **28**, 36-42 (1950).

±5 per cent for 0.02-0.06 mg. of pyruvate. Water and reagents should be redistilled from glass.

Sample—*Tissue.* To 10 mg. of wet tissue add 1 ml. of ice-cold buffer for pH 7.4. This contains 1.72 per cent of dipotassium phosphate adjusted to the desired pH with 30 per cent potassium hydroxide solution. Homogenize in the cold.

Procedure—To 0.05-0.1 ml. of homogenate add the sample of coenzyme solution and dilute to 0.2 ml. with the buffer solution.

Prepare a buffered aspartic acid solution by dissolving 1.33 grams of *dl*-aspartic acid and 0.86 gram of dipotassium phosphate in water, adjusting to pH 7.4 with potassium hydroxide solution and diluting to 50 ml.

Add 0.5 ml. to the sample. Warm the mixture to 37° and add, with mixing, 0.2 ml. of 1.46 per cent solution of α-ketoglutarate at 37°, adjusted to pH 7.4 with 30 per cent potassium hydroxide solution. Incubate for 10 minutes, add 0.1 ml. of 10 per cent trichloroacetic acid, and shake to precipitate proteins. Add 0.1 ml. of freshly prepared aniline citrate containing 50 grams of citric acid in 50 ml. of water, plus 50 ml. of aniline. Shake and incubate at 37° for about 10 minutes for development of the color. Prepare control solutions of tissue to include one stopped with trichloroacetic acid at zero time and the other incubated for 10 minutes without addition of α-ketoglutarate.

Add 1 ml. of 2,4-dintrophenylhydrazine in 100 ml. of 1:4 hydrochloric acid. Heat at 37° for 5 minutes. Add 2 ml. of toluene saturated with water and shake. Let stand 5 minutes for excess toluene to separate; centrifuge if necessary. Add 1 ml. of the toluene layer to 5 ml. of a 2.5 per cent solution of potassium hydroxide in ethanol. Mix and let stand for 5-10 minutes. Add 1 ml. of water and read against the sample blank.

TRYPSIN

The method for determining pepsin by the dye it releases by digestion of dyed fibrin is also applicable to trypsin. The necessity of using an alkaline medium with trypsin causes a difference in the color produced from fibrin stained with the same dye. The color from alcohol-blue fibrin is blue in alkaline solution.[91]

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91 A. Palladin, *Arch. ges. Physiol.* (*Pflügers*) **134**, 337-64 (1910); W. Waldschmidt, *Ibid.* **143**, 189-229 (1911); J. A. Smorodinzew and A. N. Adowa, *Biochem. Z.* **153**, 14-8 (1924).

The hydrolysis of denatured hemoglobin to give products not precipitated by trichloroacetic acid is also a measure of the tryptic activity.[92] This method is also similar to one for pepsin. The undigested hemoglobin is precipitated by trichloroacetic acid and that digested is estimated by the phenolic reaction of its tyrosine content. Unless sufficient alkalinity is present, denatured trypsin must be absent or it will become active. One unit of trypsin produces, in 6 ml. of hemoglobin digestion mixture at 35.5°, an amount of phenolic substance not precipitated by trichloroacetic acid which gives the same color as 1 milliequivalent of tyrosine when the amount of digestion is small.

Trypsin itself is a protein and, when denatured, the reaction is reversible. When the denaturing is reversed, the activity is completely restored. Special technic is therefore required for estimation of active trypsin in the presence of denatured trypsin. Urea is the denaturing agent but is not absolute in its effect; a 5 per cent increase in urea decreases digestion by 2 per cent and a 5 per cent decrease in urea increases digestion by 2 per cent. The amount of digestion is also altered by other factors such as changes in acid or alkali concentration.

Digestion of buffered casein[93] or albumin[94] is measured turbidimetrically after development with quinidine. Another method of estimation of trypsin is by alteration of pH of a peptone solution by amino acid as hydrolysis proceeds.[95]

Procedure—*By dyed fibrin.* Prepare the fibrin as for estimation of pepsin (page 515); using alcohol-blue in glycerol. For use in alkaline solution, remove fibrin from the dye solution and wash with water. Digest about an hour in a 0.1 per cent sodium bicarbonate solution, rinse with the same solution, press, and cut in fine pieces with seissors.

To a 0.3-gram portion of this fibrin add 12.5 ml. of sample and 2.5 ml. of water. Read at the end of 5 minutes or at the end of a definite period of time when the color development is satisfactory.

By hemoglobin. Denatured trypsin absent. Add water to corpuscles

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[92] M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.* **17**, 151-7 (1933); R. Kohn, H. Shay, and J. Gershon-Cohen, *J. Lab. Clin. Med.* **27**, 835-9 (1942); Rudolf Abderhalden, *Fermentforsch.* **17**, 583-81 (1945).

[93] P. Rona and Hans Kleinmann, *Biochem. Z.* **169**, 320-43 (1918); *Klin. Wochschr.* **6**, 1174-7 (1927); B. J. Krijgsman, *Z. physiol. Chem.* **228**, 256-67 (1934); I. H. Leubner, *Arch. Verdauungs-Krankl.* **63**, 14-36 (1936).

[94] M. H. F. Friedman, *Exptl. Med. and Surg.* **6**, 149-55 (1948).

[95] W. M. Persson, *Arch. intern. pharmacodynamie* **46**, 249-67 (1933).

from defibrinated bovine blood until the solution contains 10.5 grams of hemoglobin per 100 ml. This can be frozen for storage. For use mix 220 ml. of the hemoglobin solution and 11 ml. of 4 per cent sodium hydroxide solution. Heat to 50° and mix with 1300 ml. of water at 100°. Add with vigorous mixing 26 ml. of a solution containing 29.23 per cent of sodium chloride and 6.8 per cent of monopotassium phosphate. Filter and wash the precipitate with water. Separate the precipitate from the paper and add water to make a total of 400 grams. Add 400 grams of urea and 160 ml. of 4 per cent sodium hydroxide solution. When solution is complete, add 200 ml. of 13.6 per cent monopotassium phosphate solution and 240 ml. of water. Add toluene as a preservative.

Add 1 ml. of the sample enzyme solution to 5 ml. of the prepared hemoglobin. Mix and place in a water bath at 25° for 5 minutes. At once add 10 ml. of 5 per cent trichloroacetic acid solution. Mix and filter after 5 minutes. Mix 5 ml. of the filtrate, 10 ml. of 2 per cent sodium hydroxide solution, and 3 ml. of phosphotungstic-phosphomolybdic acid reagent (Vol. III, page 116), diluted to one-third concentration. Read at 550 mμ after 10 minutes.

Denatured trypsin present. Centrifuge defibrinated bovine blood and siphon off the serum and white corpuscles. Wash the corpuscles once with 0.9 per cent sodium chloride solution. Add water to give a solution containing 10.5 grams of hemoglobin per 100 ml. Freeze for storage. For use add 150 ml. of the hemoglobin solution and 7.5 ml. of 4 per cent sodium hydroxide solution at 50-60° to 90 ml. of water at 100°. Add with mechanical stirring 18 ml. of a solution containing 29.23 per cent of sodium chloride and 6.8 per cent of monopotassium phosphate. Filter and wash the filter. Dilute the filtrate with water to 390 grams. Add 390 grams of urea and 20 ml. of 4 per cent sodium hydroxide solution. Adjust to room temperature and add 160 ml. of 3.1 per cent boric acid solution and 430 ml. of water. Store at 5° with toluene as preservative. The solution can be kept for 2 months.

Complete as for trypsin absent, starting at "Add 1 ml. of the sample enzyme solution . . . ."

Nephelometrically. Prepare a mixture of 5 ml. of neutralized 1 per cent casein solution, 10 ml. of a solution containing 85 per cent of 0.947 per cent anhydrous disodium phosphate and 15 per cent of 0.92 per cent monosodium phosphate monohydrate, and 35 ml. of water. Incubate 1 ml. of sample with 10 ml. of this at 38° for 30 minutes. Add 25 ml. of water and 5 ml. of 3 per cent aqueous quinidine hydrochloride solu-

tion. Dilute to 45 ml. and read the turbidity against the same composition without enzyme.

Urease

When urease acts on urea in a phosphate buffer to form ammonium carbonate, the time to raise the pH from 6.7 to 7.7 as measured by phenol red indicates the urease activity.[96] A unit of urease activity, the Sumner unit, is the amount capable of producing 1 mg. of ammonia nitrogen in 5 minutes at 20° in a urea solution buffered with phosphate.[97] The definition implies urease activity under conditions which maintain the pH and substrate concentration within limits compatible with maximum enzyme activity and which protect the enzyme from inactivation. The number of units per mg. of preparation is equal approximately to the percentage of crystalline urease present.

Procedure—Prepare the buffered urea solution of pH 6.7 by dissolving 3 grams of urea in 10 ml. of 13.8 per cent monosodium phosphate monohydrate solution, 10 ml. of 17.4 per cent dibasic potassium phosphate solution, and enough water to make 100 ml. Also prepare a control buffer for pH 7.7 which contains 1 ml. of the sodium phosphate solution and 7 ml. of the potassium phosphate solution diluted to 80 ml.

To 5 ml. of buffered urea solution and to 5 ml. of the control buffer add 2 drops of 0.1 per cent phenol red indicator solution. Bring to room temperature and note the temperature. Add 0.5 ml. of an estimated 10 per cent urease solution to each tube, mix, and start the stop-watch. Note the time required for the urea plus enzyme to reach the same red as the control. If the colors match in less than 2 minutes, repeat the determination with urease solution formed by diluting 1 volume of the estimated 10 per cent solution to 2 volumes with water. V is then 2 instead of 1.

Calculation follows:

$$\text{Sumner units per mg.} = \frac{\text{temperature factor} \times V}{\text{time in minutes}}$$

Temperature factors are given in Table 15.

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[96] Donald D. Van Slyke and Reginald M. Archibald, *J. Biol. Chem.* 154, 623-42 (1944).

[97] J. B. Sumner and V. A. Graham, *Proc. Soc. Exp. Biol. and Med.* 22, 504-6 (1925).

TABLE 15. FACTORS FOR COLORIMETRIC TIMING METHOD

Tempertaure	*Factor*	*Temperature*	*Factor*
° C.		*° C.*	
5	2.10	25	0.758
		26	0.727
10	1.62	27	0.689
11	1.54	28	0.653
12	1.47	29	0.620
13	1.40		
14	1.33	30	0.590
		31	0.562
15	1.26	32	0.536
16	1.20	33	0.510
17	1.14	34	0.485
18	1.08		
19	1.03	35	0.461
20	0.982	40	0.363
21	0.937		
22	0.890	45	0.304
23	0.842		
24	0.800	50	0.270
		55	0.245

XANTHINE OXIDASE

This enzyme is determined by periodic measurements to show the rate of disappearance of the substrate, xanthine.[98] The latter is measured after reaction with the phenol reagent. Each micromole of xanthine disappearing per hour per gram of liver corresponds to an enzyme unit. Other purines such as adenine and guanine give the color reaction, but this effect is compensated for by the blank.

Recovery of added xanthine differed from the calculated by ±6 per cent.

Sample—*Rat livers.* Homogenize weighed portions of fresh chilled liver in 5 volumes of 0.039 *M* sodium-potassium phosphate buffer at pH 7.4 (Vol. I, page 174) and strain through gauze. Incubate 5-ml. portions of homogenate for 40 minutes at 37° in a shaker. Add 0.3 ml. of buffer solution and 0.6 ml. of water to the control flask. To the test flask add 6 ml. of 0.58 per cent xanthine solution in dilute alkali. The xanthine is dissolved by gentle heating. Mix and remove a 1-ml. aliquot

[98] G. Litwack, J. W. Bothwell, J. N. Williams, Jr., and C. A. Elvehjem, *J. Biol. Chem.* **200**, 303-10 (1953).

from each for the zero time reading. Continue the shaking and remove 1-ml. samples for reading every 30 minutes for a total of 2 hours. If homogenates are of low activity, take aliquots at 1-hour intervals for a total of 4 hours.

Pipet the 1-ml. aliquots into a mixture of 1 ml. of 40 per cent sodium tungstate solution, 5 ml. of water, and 1 ml. of 1:15 sulfuric acid, to precipitate proteins. Dilute each to 10 ml. and transfer to a centrifuge tube, washing with 2 ml. of water. Centrifuge and use the supernatant liquid as sample.

Procedure—To 0.5 ml. of sample solution add 2.5 ml. of water and 1 ml. of 1:1 phosphotungstic-phosphomolybdic acid reagent (Vol. III, page 116). Add 5 ml. of a saturated sodium carbonate solution. Read the solutions at 660 mμ against a reagent blank.

CHAPTER 13

ANTIBIOTICS [1]

The antibiotics are classified here even though many have such pronounced chemical structure as to have justified classification by it. One justification for the present classification, if one is needed, is that they are not only prepared by similar methods, but some of the methods of determination have much in common.

Penicillins

Penicillins in general are complex heterocyclic acids in which substituents may vary. The reaction with hydroxylamine at an amide portion of the structure forms hydroxamic acid, breaking the—CO—N— bond. This reaction for acylphosphates [2] is read as the purple ferric complex.[3] To correct for interference, penicillin is converted to penicilloic acid with the enzyme penicillinase in another portion and similarly developed. This gives all the background color of the first sample with none of the penicillin. The reaction is a general one as it is given by acids, amides, and anhydrides. The reaction with aldehydes or ketones gives oximes which give colored complexes with iron. The diversity of these emphasizes the need for a sample blank. Also, the color fades, necessitating careful control of the time of development. Chilling the developed sample will slow the rate of fading.[4] The color is roughly a molar function with at least penicillins G, X, F, and K and does not entirely conform to Beer's law. Phosphates reduce the color intensity. Alkali inactivation is not suitable for preparation of the blank used here; it liberates reactive material. Accuracy to ±5 per cent is to be expected.

The color developed by reaction with N-(1-naphthyl-4-azobenzene) ethylenediamine is also appropriate.[5] Reasonable variations of time and

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[1] See Volume III, Chapter 1, for details of organization, condensation, etc.

[2] Fritz Lipmann and L. Constance Tuttle, *J. Biol. Chem.* **159**, 21-8 (1945).

[3] Jared H. Ford, *Anal. Chem.* **19**, 1004-6 (1947); George E. Boxer and Patricia M. Everett, *Ibid.* **21**, 670-3 (1949).

[4] Toju Hata and Yoshimoto Sano, *Kitasato Arch. Exptl. Med.* **22**, 27-32 (1949).

[5] John V. Scudi, *J. Biol. Chem.* **164**, 183-94 (1946); A. L. Yaroslavtsev and A. N. Klimov, *Zhur. Obshcheĭ Khim.* **20**, 2279-83 (1950).

temperature do not affect the result. Some loss occurs by agglomeration of the colored condensation product at interfaces during extraction. The method will estimate 0.001 mg. Inactivation products do not react. For increased sensitivity by fluorometry, penicillin is condensed with 2-methoxy-6-chloro-9-(β-aminoethyl)aminoacridine.[6] A sample containing 0.0000625-0.000625 mg. of penicillin G per ml. can be read to ± 10 per cent.

Penicillin can be nitrated, reduced to the amine, diazotized, and coupled with N-(1-naphthyl)ethylenediamine dihydrochloride and read.[7] Penicillin F does not react and many aromatic impurities do. Penicillin hydrolyzed with acid and heat gives penicillamine suitable for estimation by Nessler's reagent.[8] The ninhydrin reaction (page 107) is also applicable to penicillin.[9]

Penicillin G, X, K, and F in 0.4 *M* acetate buffer for pH 4.6 gives no absorption at 290-360 mμ. After 15 minutes at 100° the reaction product shows a maximum at 322 mμ which will read 0.005-0.075 mg. per ml. to ± 5 per cent.[10] An alternative method of estimation is turbidimetric from the effect on growth of streptococci [11] or staphylococci.[12] The lactic acid formed by *Lactobacillus bulgaricus* is also a method of estimation.[13] Yet another is the nitrite produced from nitrate by *Staphylococcus aureus*, the production being proportional to the growth of the organism.[14] The nitrite is then developed with N-(1-naphthyl) ethylenediamine dihydrochloride.

Sample—*Penicillin salts.* Sterilize a 0.0025 *M* phosphate buffer for pH 7 (Vol. I, page 176) by autoclaving for 1 hour at 15 pounds pressure. Dissolve the penicillin salt in this and dilute to 0.00025-0.002 *M* for development by hydroxylamine.

Fermentation liquors. Filter through paper and develop by hydroxylamine.

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[6] John V. Scudi and Viola C. Jelinek, *J. Biol. Chem.* **164**, 195-201 (1946).

[7] J. E. Page and F. A. Robinson, *Nature* **158**, 910-11 (1946).

[8] G. Del Vecchio and R. Argenziano, *Boll. soc. ital. biol. sper.* **22**, 1189-90 (1946).

[9] M. J. Carl Allinson, *Proc. Soc. Exptl. Biol. Med.* **60**, 293 (1945).

[10] Roger M. Herriott, *J. Biol. Chem.* **164**, 725-36 (1946).

[11] S. W. Lee, E. J. Foley, Jeanne A. Epstein, and J. H. Wallace, Jr., *Ibid.* **152**, 485-6 (1944).

[12] H. Velu and J. Mainil, *Compt. rend. soc. biol.* **141**, 470-1 (1947); Kiyoshi Higuchi and W. H. Peterson, *Anal. Chem.* **19**, 68-71 (1947).

[13] Georges Sanchez and Adre Lamensens, *Compt. rend.* **224**, 1189-91 (1947).

[14] Andres Goth and Milton T. Bush, *Ind. Eng. Chem., Anal. Ed.* **16**, 451-2 (1944).

Urine. Acidify 100 ml. to pH 2 with phosphoric acid and extract with several successive portions of ether. Extract the combined ether extracts with 10 ml. of 0.85 per cent sodium bicarbonate solution. Acidify the aqueous alkaline extract with 1:18 sulfuric acid and heat until hydrolysis is complete. Cool and filter. Add 20 per cent sodium hydroxide to definite alkalinity. Determine by Nessler's reagent (page 181).

Procedure—*By hydroxylamine.* As a sample blank treat 1 ml. of sample solution with 1 drop of penicillinase A solution containing about 10,000 units per ml. Let it stand for 10 minutes at room temperature. Prepare a fresh neutralized alcoholic hydroxylamine solution by mixing 1 volume of 35 per cent aqueous hydroxylamine hydrochloride, 1 volume of 17.7 per cent aqueous sodium hydroxide containing 2 per cent of anhydrous sodium acetate, and 4 volumes of ethanol. Add 3 ml. of this solution to the sample and blank and mix.

Prepare a ferric reagent containing 100 grams of ferric ammonium sulfate dodecahydrate and 46.7 ml. of concentrated sulfuric acid per 500 ml. Add 1 ml. of this to the sample and blank after 3 minutes. Mix and read within 2-5 minutes at 515 mμ, using the sample blank to set the zero point of the instrument.

With N-(1-naphthyl-4-azobenzene)ethylenediamine. As reagent dissolve 12.5 grams of aniline hydrochloride in 625 ml. of water containing 16 ml. of concentrated hydrochloric acid. Cool to approximately 0° and add dropwise 8 grams of sodium nitrite in 125 ml. of water. After 15 minutes add ammonium sulfamate to destroy excess nitrite until there is no further reaction with starch iodide paper. Add this cold diazonium salt solution with stirring to 25 grams of N-(1-naphthyl) ethylenediamine dihydrochloride in 1250 ml. of cold water containing 40 ml. of concentrated hydrochloric acid. Let stand at room temperature for 10-15 minutes and warm to 50-60°. Chill after 30 minutes and add 10 per cent sodium hydroxide solution to make alkaline so that the color changes from deep purple to orange. Filter the precipitate and dry. Reflux the ground precipitate with 2500 ml. of 85-100° petroleum ether. Filter while hot and chill the filtrate to obtain the product. Recrystallize as 1 per cent solution in petroleum ether. For use as reagent prepare a 0.07 per cent solution in benzene.

Prepare a glycine buffer of 60 ml. of 1.5 per cent glycine solution in 11.7 per cent sodium chloride solution and adjust to pH 2 with 540 ml. of 1:50 hydrochloric acid. Mix 5 ml. of this with 25 ml. of

chloroform, chill to below 5°, and add 5 ml. of aqueous sample containing 0.02-0.12 mg. of test substance, similarly cooled. Shake for 2 minutes and let separate. To the separated chloroform layer in a prechilled graduate, add 3 grams of anhydrous sodium sulfate. Mix 15 ml. of the reagent solution with 20 ml. of the chloroform extract and immediately add 5 ml. of a 0.5 per cent solution of acetic acid in benzene. After 3 hours ±3 minutes in a closed container, extract for 10 seconds with 25 ml. of 0.2 per cent sodium hydroxide solution. Separate the lower layer incompletely so as not to disturb the interface. Extract the sodium hydroxide solution with a further 25 ml. of chloroform and separate, leaving 0.2-0.3 ml. of solvent behind. Extract the combined chloroform extracts with 1 ml. of concentrated hydrochloric acid and 15 ml. of 1:4 butanol-benzene for 10 seconds. The red condensation product is now in the butanol-beneze phase. Mix 10 ml. of this extract with 2 ml. of 5:1 concentrated hydrochloric acid-absolute ethanol and read at 540 mμ.

Fluorometric. For preparation of the reagent, warm 5 grams of 6,9-dichloro-2-methoxyacridine (halocrin) with 100 ml. of anhydrous ethylenediamine for 20 minutes at 100°. The solid dissolves in 15 minutes or less. Boil for 3 minutes and cool to room temperature. Vacuum-distil about 60 ml. of ethylenediamine and add 250 ml. of water to the cooled residue. Add 500 ml. of 1:120 hydrochloric acid to dissolve the precipitate and filter. Add 30 per cent sodium hydroxide solution to maximum precipitation. Filter, dry, and recrystallize from an approximately 0.2 per cent solution in low-boiling petroleum ether.

To 5 ml. of 0.2 per cent solution of the reagent, add 2 ml. of acetone, 10 ml. of sample in chloroform, and 5 ml. of 1 per cent acetic acid in benzene. After 60 ±5 minutes in the dark, shake for 10 seconds with 10 ml. of 2 per cent sodium hydroxide solution. Separate and discard the organic layer. Extract with two successive 5-ml. portions of chloroform and discard the extracts. Add 1 ml. of acetic acid to the aqueous layer and extract with 15 ml. of 1:2 butanol-benzene solution by shaking for 30 seconds. Discard the aqueous layer and wash the extract for 30 seconds with 10 ml. of 1:20 acetic acid. Discard these washings and add 50 ml. of chloroform and 15 ml. of 2 per cent sodium hydroxide solution. Shake for 30 seconds to transfer the product to the alkaline solution and separate it. Add 1 ml. of concentrated hydrochloric acid and read the fluorescence with riboflavin filters.

Benzylpenicillin, Penicillin-G

Benzylpenicillin is determined by nitration and reduction with hydroxylamine.[15] The solution conforms to Beer's law over a reasonable range. Close attention to detail is essential. Penicillins K and F give no reaction. Penicillin X, *p*-hydroxybenzyl penicillin, interferes. Accuracy to ±3 per cent is obtained other than on fermentation broths and ±5 per cent then. Nitrated benzylpenicillin is extracted from the neutralized nitration mixture into butanone and read at 530 mμ.[16]

Direct reading of the absorption of the phenylacetyl side chain in the ultraviolet at 263 mμ has been used.[17] More specifically it is read by the difference in absorption at a peak of 264.5 mμ and the dip of 263 mμ. Proportionality exists over the range 0.001-0.01 *M*.[18]

Penicillin G is determined in penicillin O by oxidation with alkaline permanganate to benzoic acid[19] and reading in ammonical solution in the ultraviolet.[20] The recovery in the procedure is 86 ±4 per cent, except for 2-chloroprocaine penicillin where it is 78 ±4 per cent. The loss is of benzoic acid in oxidation. The loss is much greater if the alkaline permanganate is acidified before reduction of excess and precipitated manganese dioxide with oxalic acid.

Sample—*Penicillin.* Prepare a solution containing 0.2-1 mg. per ml. Measure two 5-ml. portions. To the blank add 1 ml. of 8 per cent sodium hydroxide solution and mix. Immerse in boiling water for 1 minute and cool. Add 4 ml. of 1:20 hydrochloric acid and mix. Add

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[15] George E. Boxer and Patricia M. Everett, *Anal. Chem.* **21**, 670-3 (1949); Cf. G. Del Vecchio and R. Argenziano, *Boll. soc. ital. biol. sper.* **22**, 1190-2 (1946); J. E. Page and F. A. Robinson, *Nature* **158**, 910-11 (1946); Dorothy J. Hiscox, *Anal. Chem.* **22**, 722-3 (1950).

[16] Teodor Canbäck, *Farm. Rev.* **46**, 97-101 (1947).

[17] Thomas C. Grenfell, John A. Means, and Ellis V. Brown, *J. Biol. Chem.* **170**, 527-35 (1947); A. R. Philpotts, W. Thain, and G. H. Twigg, *Nature* **159**, 839-40 (1947); A. A. Colon, F. G. Herpich, J. D. Neuss, and H. A. Frediani, *J. Am. Pharm. Assoc.* **38**, 138-42 (1949).

[18] Gabor B. Levy, Denman Shaw, Eldon S. Parkinson, and David Fergus, *Anal. Chem.* **20**, 1159-61 (1948); Cf. H. Pénau, G. Hagemann, Y. Leclere, and R. Viennet, *Ann. pharm. franç.* **8**, 450-6 (1950).

[19] A. R. Philpotts, W. Thain, and G. H. Twigg, *Nature* **159**, 839 (1947).

[20] J. L. Johnson, W. A. Struck, E. J. Scott, and J. E. Stafford, *Anal. Chem.* **25**, 1490-2 (1953).

5 ml. of water to the sample. Carry 5 ml. of each through the procedure by nitration and reduction.

Potassium penicillin O. Mix a 0.3-gram sample with 1 ml. of 2.5 per cent potassium hydroxide solution. Add 50 ml. of 4.5 per cent potassium permanganate solution and reflux for 1 hour with precautions against bumping. Rinse the condenser, cool to room temperature, and add 25 ml. of 10 per cent oxalic acid solution. After the initial reaction abates, add 15 ml. of 1:1 sulfuric acid in several portions with mixing and cooling to keep below 25°.

For subsequent extraction use Teflon plugs or white petrolatum as lubricant. To the clear colorless solution add 30 grams of sodium chloride. Extract with 30, 20, and 10 ml. of chloroform and filter the extracts through glass wool. Add exactly 50 ml. of 0.1 N ammonium hydroxide to the combined chloroform extracts and shake for 5 minutes. Discard the chloroform and filter the ammoniacal extract through glass wool for reading as benzoic acid.

Procaine penicillin O and 2-chloroprocaine penicillin O. Dissolve a 0.5-gram sample in 50 ml. of chloroform and extract with 50 ml. of 1:4 ammonium hydroxide. Discard the chloroform and filter the ammoniacal solution through glass wool. Use 20 ml. of water to wash during the transfer. Add 3 ml. of 2.5 per cent potassium hydroxide solution and boil vigorously down to about 20 ml., with precautions against bumping. Cool, add 2.5 grams of potassium permanganate slowly, and reflux for 1 hour. Complete as for potassium penicillin O from "Rinse the condenser, cool to . . ." but add 30 ml. of 10 per cent oxalic acid instead of 25 ml.

Broth. Dilute to 0.025-0.15 mg. of penicillin G per ml. As glycine buffer for pH 1.5 dissolve 45 grams of glycine, 35 grams of sodium chloride, and 45 ml. of concentrated hydrochloric acid per liter. Combine 60 ml. of sample, 60 ml. of the buffer, and 120 ml. of amyl acetate in a separatory funnel. Shake vigorously for exactly 2 minutes and let the phases separate over 2-3 minutes. Discard the aqueous phase, leaving the interface in the funnel. Decant most of the clear amyl acetate layer, free from interface, and dry superficially by shaking with about a gram of anhydrous sodium sulfate for a few seconds. Shake exactly 100 ml. of the chilled amyl acetate layer with 15 ml. of ice-cold 2.6 per cent dipotassium phosphate solution for 1 minute, separate the aqueous layers and keep cold.

Shake 5 ml. of the cold aqueous extract with 5 ml. of prechilled

1:35 hydrochloric acid and 25 ml. of ice-cold chloroform for 2 minutes. Separate as the sample to carry through the procedure by nitration and reduction. Combine 6 ml. of aqueous extract with 3 ml. of 7.2 per cent sodium hydroxide solution and place in boiling water for 1 minute. Cool and add 3 ml. of 1:5 hydrochloric acid. Mix and shake a 10-ml. aliquot with 25 ml. of chloroform for 2 minutes. This is the blank to be carried through the procedure.

Procedure—*By nitration and reduction.* For the operations use chilled glassware and have stopcocks rinsed with chloroform and lubricated with distilled water just before use. All solutions and solvents are refrigerated. As buffer for pH 2 dissolve 9 grams of glycine, 7 grams of sodium chloride, and 9 ml. of concentrated hydrochloric acid in water and dilute to 1 liter. There will be a prepared sample of penicillin in 25 ml. of chloroform and a prepared blank in which the penicillin has been degraded to non-reactive form. Each is carried through the following procedure.

Transfer the 25 ml. of sample or blank in solvent into a 25-ml. glass-stoppered graduated cylinder containing about 0.5 gram of anhydrous sodium sulfate. Shake a few seconds to dry and decant. This prevents water separation during subsequent evaporation. Pipet exactly 20 ml. into a 50-ml. glass-stoppered conical flask, add a glass bead, and evaporate to dryness on a steam bath. Not over 100° is permissible. Add 1 ml. of 10 per cent potassium nitrate solution in concentrated sulfuric acid and rotate to insure contact with all dried residue. Replace on the steam bath for 30 minutes, stoppering after the first minute. When nitration is complete, chill in an ice bath. Incomplete heating gives an unstable color; heating too long lessens the stable color.

Add 2 ml. of cold water cautiously along the walls, mix, and cool to 0°. Add 2 ml. of concentrated ammonium hydroxide dropwise along the walls and mix while still in a bath of ice and water. Remove and let rise to room temperature. Add 2 ml. of 15 per cent aqueous hydroxylamine solution and mix. Add 5 ml. of concentrated ammonium hydroxide and mix. After 45 minutes at room temperature, read at 580 mμ against distilled water. The color is stable at least 2 hours. Subtract the reading for the blank in which penicillin has been destroyed, but which has otherwise been carried through the identical procedure.

As benzoic acid. Read the prepared solution at 220 and 224 mμ against a blank of 0.1 N ammonium hydroxide shaken with chloroform.

To maintain saturation with chloroform keep a few drops in the container with the sample and blank until transferred to the cuvet.

Procaine Penicillin

Procaine penicillin is read in chloroform at 5.6 mμ.[21]

Citrinin

Citrinin, a penicillin adjunct, gives a brown with ferric ion which on dilution turns to yellow and then to light blue.[22]

Sample—*Urine.* Extract an acidified sample containing up to 0.5 mg. of citrinin with 10 ml. and 10 ml. of benzene. This leaves interfering chromogens behind. Wash the benzene extract with 5 ml. and 5 ml. of water and discard the aqueous layers. Extract the citrinin from the benzene into 10 ml. of 1 per cent sodium bicarbonate solution. Discard the benzene layer and first acidify the sodium bicarbonate solution; then extract with 5 ml. of benzene. Wash the benzene with 5 ml. of water and filter through a dry paper. Evaporate the benzene filtrate and take up in 2 ml. of ethanol.

Procedure—Add 0.5 ml. of 0.15 per cent aqueous ferric chloride solution and dilute to 5 ml. with water. Read at once if pure blue at 555 mμ against a reagent blank. If not entirely blue, dilute to a known volume with water.

Terramycin

Terramycin is determined by its ultraviolet absorption in acid solution.[23] This corresponds to a similar method for aureomycin and gives results reproducible to about ±4 per cent and agreeing with those by bioassay, to about ±6 per cent.

Another method is by the color produced with ferric chloride in acid solution,[24] by the disappearance of a characteristic band in the ultraviolet measured by the action of dilute alkali on an originally acid

[21] N. H. Coy, C. W. Sabo, and B. T. Keeler, *Anal. Chem.* **21**, 669-70 (1949).

[22] Yu Wang and Hung Shun Ting, *Science and Technology in China* 1, 23-4 (1948).

[23] Dorothy J. Hiscox, *J. Am. Pharm. Assoc.* **40**, 237-40 (1951).

[24] F. Monastero, John A. Means, T. C. Grenfell, and F. Howard Hedger, *Ibid.* **40**, 241-5 (1951).

solution, and by the absorbance of an alkaline solution. This method is applicable for determination of 0.05-1 mg. of terramycin. It is accurate to ±2 per cent and agrees with microbiological results to about ±3 per cent. The method using the difference in absorbance between acid and alkaline solutions is applicable for 0.015 mg. per ml. or more of terramycin. Measurement of the absorbance of an alkaline solution is applicable to solutions containing about 0.5 mg. of terramycin per ml. It gives results accurate to about ±5 per cent and about 17 per cent higher than those obtained microbiologically. The turbidimetric technic for estimation of antibiotics is applied to terramycin with *Klebsiella pneumoniae*.[25] Results are reproducible to 2 per cent.

Samples—For reading in the ultraviolet or by ferric chloride, prepare samples as in the method for aureomycin (page 555).

Liquids. Dilute to 0.0005-0.0025 mg. of terramycin per ml. and develop turbidimetrically.

Free base. Dissolve in 1:120 hydrochloric acid and dilute as for liquids for turbidimetric development.

Urine. Centrifuge if turbid, dilute, and develop turbidimetrically.

Whole blood. Centrifuge and dilute with clear serum for development turbidimetrically.

Procedure—*For ultraviolet reading in acid solution.* To an aqueous solution containing 0.025-0.25 mg. of terramycin in 3 ml. add 3 ml. of 1:35 sulfuric acid. Insert a foil-wrapped stopper carrying an air condenser. Heat in boiling water for 30 minutes. Cool and dilute to 25 ml. with water. Read at 249 and 312 mμ. The terramycin is proportional to the difference in absorbance.

By ferric chloride. Acidify a solution of terramycin with concentrated hydrochloric acid to pH 2. Dilute a volume of acidified solution containing 0.5-1 mg. of terramycin to 10 ml. with 1:120 hydrochloric acid. Add 10 ml. of 0.05 per cent ferric chloride in 1:120 hydrochloric acid and mix. Let stand for 10 minutes and read at 490 mμ against a reagent blank. The color is stable for 2 hours.

By reading in both acid and alkaline solutions. As the acid solution, dilute 10 ml. of sample containing 0.5 mg. per ml. of terramycin to 250 ml. with 1:120 hydrochloric acid. As the alkaline solution dilute 2 ml. of solution containing 1 mg. of terramycin in 1:1200 hydrochloric acid

[25] R. C. Kersey, *Ibid.* **39**, 252-3 (1950).

with 5 ml. of water. Add 5 ml. of 1.6 per cent sodium hydroxide solution and immerse in boiling water for 5 minutes. Cool for 2 minutes with ice water and dilute to 20 ml. Dilute 10 ml. of this solution to 25 ml. with 1:80 hydrochloric acid. The difference in reading between the acid solution and the alkaline solution read at 353 mμ, furnishes the concentration of terramycin. By use of more concentrated reagents, this method can be applied to solutions containing about 0.015 mg. per ml.

By reading in alkaline solution. Treat the sample as under the alkaline solution through ". . . dilute to 20 ml." Read against a water blank at 440 mμ.

Turbidimetrically. Prepare a stock culture of *Klebsiella pneumoniae* PCI 602 on agar slants of 0.15 per cent of beef extract, 0.3 per cent of yeast extract, 0.6 per cent of peptone, and 1.5 per cent of agar at pH 7. Transfer the organism to fresh agar slants and incubate for 6 hours at 37°. Suspend the growth from 2-3 slants in sterile water and add 5 ml. of the suspension to 2 Roux bottles containing the agar mixture. Incubate at 37° for 6 hours and harvest the growth. Add sterile water to a reading of 80 per cent at 650 mμ.

As assay medium prepare 0.5 per cent peptone, 0.15 per cent yeast extract, 0.15 per cent beef extract, 0.35 per cent sodium chloride, 0.1 per cent glucose, 0.132 per cent monopotassium phosphate, and 0.368 per cent dipotassium phosphate at pH 7 and sterilize.

Pipet into a series of tubes 0.04 to 0.2 ml. of sample at 0.04-ml. intervals. Inoculate sufficient sterile assay broth with 6 ml. of the cell suspension per 100 ml. Add 7 ml. of the inoculated medium to each tube. Incubate at 37° for 210 minutes and then steam for 10 minutes to prevent further growth. Read at 650 mμ for comparison with a standard curve.

Chloromycetin, Chloroamphenicol

The familiar coupling with an amine is applicable to chloromycetin. For this the aryl nitro group is reduced to an amine with titanous chloride,[26] stannous chloride,[27] or zinc dust.[28] This is then diazotized

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[26] Anthony J. Glazko, Loretta M. Wolf, and Wesley A. Dill, *Arch. Biochem.* 23, 411-18 (1949).

[27] Samuel P. Bessman and Sara Stevens, *J. Lab. Clin. Med.* 35, 129-35 (1950).

[28] Makota Oda and Minoru Hirano, *J. Pharm. Soc. Japan* 71, 51-2 (1951); E. Ginoullriac and L. Mainardi, *Giorn. mal. infettive e parassitarie* 1, 252-6 (1949).

and coupled with N-(1-naphthyl)ethylenediamine dihydrochloride.[29] The red color is 4 times as sensitive when reduction is with stannous chloride as that from reduction with titanous chloride. Adenine, adenosine triphosphate, and nucleic acid interfere with reduction by zinc dust. Adenine is not reduced by stannous chloride. Inactive metabolic derivatives of chloromycetin interfere by any method of reduction. Diazotizable amines interfere.

The chloromycetin reduced with stannous chloride can also be diazotized and coupled with α-naphthol.[30] Chloromycetin reduced in acid solution with aluminum is diazotized and developed with β-naphthol to give a red color.[31] This is read at 400 mμ. The diazotized compound is also read as such, 2 hours after addition of the nitrite.[32] Chloromycetin is read directly at 278 mμ.

Another attack is to hydrolyze with acid and to oxidize with iodate. The resulting *p*-nitrobenzaldehyde is determined by 2,4-dinitrophenylhydrazine [33] or the corresponding dinitrobenzaldehyde. Another method depends on a reddish brown color with picric acid or 2,4-dinitrosalicylic acid.[34]

Sample—*General.* As reagent add 6 ml. of 15 per cent titanous chloride to 30 ml. of concentrated hydrochloric acid. Heat to boiling and cool under hydrogen. Dilute with 360 ml. of oxygen-free water which has also been stored under hydrogen.

Transfer two 1-ml. portions of sample containing not over 0.15 mg. of chloromycetin. To one add 2 ml. of water and 1 ml. of reagent. Mix and add 1 ml. of 4 per cent sodium hydroxide. The pH should now approximate 9. Centrifuge to throw down the precipitate of titanium compounds. To the other tube add 4 ml. of water as a sample blank. Determine by coupling.

Alternatively add 4 ml. of 1:20 hydrochloric acid to two 1-ml. samples. Add 50 mg. of zinc dust to one and heat both tubes in boiling water

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29 Joseph Levine and Henry Fischbach, *Antibiotics and Chemotherapy* 1, 59-62 (1951).

30 Togo Lassandro Pepe, *Boll. chim. farm.* **88**, 411-13 (1949).

31 Pereira Forjaz, *Anais Azevedos* (Lisbon) **1**, 163-8 (1949).

32 R. Damade and Ch. Dulong de Rosnay, *J. med. Bordeaux* **127**, 623-8 (1950).

33 Ramon Puga, *Rev. farm.* (Buenos Aires) **93**, 240-50 (1951); *Ibid.* **93**, 290-307 (1951); Herman Garcia Madrid, *Colegio farm.* **9**, No. 118, 1-4 (1952).

34 K. L. Arora and C. R. Krishna Murti, *Current Sci.* (India) **21**, 52 (1952).

for 1 hour. Cool quickly and dilute each to 1 ml. Determine by coupling.

Urine. Mix 1 ml. of urine with 2 ml. of 0.2 *M* phosphate buffer for pH 6 (Vol. I, page 176). Shake for 10 minutes with 6 ml. of ethyl acetate. Centrifuge and separate the organic solvent layer. Wash twice with an equal volume of the buffer saturated with ethyl acetate. Evaporate 5 ml. of the ethyl acetate layer to dryness on a steam bath and take up the residue in 3 ml. of water for reduction with titanous chloride, starting at "Transfer 1 ml. of sample . . ."

Serum. Mix 5 ml. of serum with 5 ml. of 0.2 *M* phosphate buffer for pH 6 (Vol. I, page 176). Extract with 25 ml. and 25 ml. of 2:1 chloroform-ethyl acetate. Filter the combined extracts and evaporate to dryness. Dissolve the residue in 3 ml. of 0.4 per cent sodium hydroxide solution for direct coupling or reduction and coupling with N-(1-naphthyl)ethylenediamine.

Urine. Use 2 ml. of urine in place of 5 ml. of serum.

Tissue homogenate. Use 2 ml. in place of 5 ml. of serum.

Procedure—*Direct coupling.* To 3 ml. of unreduced sample in 0.4 per cent sodium hydroxide solution add 25 mg. of sodium persulfate and let stand for 15 minutes at room temperature. Add 0.5 ml. of 5 per cent sodium nitrite solution and about 0.3 ml. of concentrated hydrochloric acid. After about 2 minutes add 1 ml. of 5 per cent sulfamic acid. After about 2 minutes add 0.5 ml. of 0.5 per cent N-(1-naphthyl)ethylenediamine dihydrochloride solution and dilute to 25 ml. If cloudy extract after 2 hours with 5 ml. of chloroform. Centrifuge and read the aqueous phase at 558 mμ against a reagent blank.

By coupling with N-(1-naphthyl)ethylenediamine. Add 0.5 ml. of fresh 1 per cent sodium nitrite to the prepared reduced sample and sample blank. After 2 minutes add 2 ml. of 0.5 per cent ammonium sulfamate solution in 27.6 per cent monosodium phosphate monohydrate solution. Mix and after 3 minutes add 0.5 ml. of 0.2 per cent aqueous solution of the coupling agent. Dilute to 6 ml. and read after 20 minutes at 555 mμ against a reagent blank. Correct for the sample blank.

By picric acid or 2,4-dinitrosalicylic acid. As reagent dissolve 1 gram of the acid in water with 30 grams of potassium sodium tartrate and 20 ml. of 8 per cent sodium hydroxide solution and dilute to 100 ml. Mix 2 ml. of sample and 2 ml. of reagent and heat at 100° for 10 minutes. Cool, dilute to 10 ml., and read at 530 mμ against a similarly treated blank.

Streptomycin

When streptomycin is heated in dilute alkaline solution, it forms maltol,[35] 2-methyl-3-hydroxy-γ-pyrone. This is used as the basis of a specific method.[36] On cooling, ferric ammonium sulfate or a phenol reagent is used to develop color from the maltol. To separate from interfering substances in broth or urine, a double extraction with chloroform from an acid medium is required. Then the maltol is extracted from the chloroform with dilute aqueous alkali and developed. Emulsions may interfere.

The method does not distinguish streptomycin from the mannisidostreptomycin which is only 15-20 per cent as active. Results are reproducible to ±2 per cent. Each is determined by getting the sum as maltol on an eluted sample and then correcting for the mannisidostreptomycin as determined with anthrol.[37] The color on oxidation with periodic acid is determined along with that for mannisidostreptomycin and the amount of each solved by simultaneous equations, the method being given under the latter topic.

When aqueous streptomycin is heated at 100° with acetyl acetone and sodium hydroxide, it forms the amine. This reacts with *p*-dimethylaminobenzaldehyde to give a pink readable at 540 mμ.[38] Streptomycin gives a red color with l-naphthol and sodium hypobromite suitable for reading at 520 mμ.[39] The carbonyl group also reacts with a highly colored semicarbazide.[40] In an acid medium 2,4-dinitrophenylhydrazine forms a yellow hydrazone with streptomycin.[41] The color is read at 430 mμ.

Streptomycin and dihydrostreptomycin are determined by the color produced with sodium nitroprusside and potassium ferricyanide in alkaline solution.[42] The decomposition product, streptidine, would inter-

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35 J. R. Schenk and M. A. Spielman, *J. Am. Chem. Soc.* **67**, 2276-7 (1945).

36 G. E. Boxer, Viola C. Jelinek, and P. M. Leghorn, *J. Biol. Chem.* **169**, 153-65 (1947); William Eisenman and Clark E. Bricker, *Anal. Chem.* **21**, 1507-8 (1949).

37 C. V. St. John, D. E. Flick, and J. B. Tepe, *Ibid.* **23**, 1289-91 (1951).

38 John V. Scudi and Viola C. Jelinek, *Science* **104**, 487 (1946); J. Masquelier, *Bull. trav. soc. Bordeaux* **87**, 53-5 (1949).

39 R. R. Buck, W. J. Mader, and H. A. Frediani, *Bol. col. quím. Puerto Rico* **6**, 6-9 (1949).

40 E. K. Marshall, Jr., K. C. Blanchard, and Emmett L. Buhle, *J. Pharm. Exptl. Therap.* **90**, 367-74 (1947).

41 E. M. Savitskaya and V. D. Kartseva, *Zhur. Anal. Khim.* **8**, 46-9 (1953).

42 F. Monastero, *J. Am. Pharm. Assoc., Sci. Ed.* **41**, 322-4 (1952).

fere but is removed as an insoluble compound. Sodium chloride in excess of 0.5 per cent in dilute solutions of the compounds reduces color intensity. The method is accurate to ±2 per cent.

A fluorometric method is based on the formation of a hydrazone with the fluorescent 9-hydrazinoacridine hydrochloride.[43] Excess reagent and neutral or acid hydrazones are extracted from the acidified solution with benzyl alcohol leaving the basic hydrazone from streptomycin in solution. Any other basic substance would interfere. The ultraviolet absorption at 420 mμ of the alkaline solution after hydrolysis is used.[44] Turbidimetric methods with test organisms are also applied.[45]

Sample—*Broth.* To prepare the chromatographic resin, stir sodium amberlite IRC-50 for 1 hour with sufficient 4 per cent sodium hydroxide solution to leave the pH of the solution at 9-10. Wash by decantation to pH 7-8. Filter, air-dry, and screen to use the 20-40 mesh fraction. To standardize, treat 1.5 grams of resin with 15 ml. of 1:50 sulfuric acid, filter, and wash with water. Titrate the combined filtrate and washings with 4 per cent sodium hydroxide to phenolphthalein. Unless the titration is 6.5 ml. of alkali ±3 per cent, adjust the weight of resin as called for in the technic. Adjust the mold-containing broth to pH 2-2.3 and filter.

Wash 1.5 grams of resin with water and then add 10 ml. of filtered broth. Add water to 20 ml. and shake mechanically for 10 minutes. Decant and wash the resin 4-5 times with water to remove color and suspended matter. The antibiotic is now in the resin, so none must be lost. Add 15 ml. of the 1:50 sulfuric acid used in standardization of the resin and shake again mechanically for 10 minutes. Filter and wash with water. Add 4 per cent sodium hydroxide solution, usually 6.5 ml., to adjust to a phenolphthalein endpoint. Dilute to 50 ml. Determine the total streptomycin and any mannisidostreptomycin as maltol by ferric ion.

When mannisidostreptomycin is present add a 35-ml. aliquot of the eluate to 1.5 grams of the prepared resin and proceed as before from ". . . shake mechanically for 10 minutes." The eluate this time is

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[43] George E. Boxer and Viola C. Jelinek, *J. Biol. Chem.* **170**, 491-500 (1947); Viola C. Jelinek and George C. Boxer, *Ibid.* **175**, 367-75 (1948).

[44] Elwood Titus and Josef Fried, *Ibid.* **174**, 57-70 (1948).

[45] Elizabeth J. Oswald and Lila F. Knudson, *J. Am. Pharm. Assoc.* **39**, 61-6 (1950).

for estimation of mannisidostreptomycin by anthrone to get the value for streptomycin by difference.

Preparation by steam distillation. The distillation apparatus required is shown in Figure 20. Transfer a sample containing 50-2500 units of streptomycin to the standard-taper tube and dilute to 4 ml. with water. Add 1 ml. of 20 per cent sodium hydroxide solution and heat in boiling water for 3 minutes. Cool to room temperature and add 2 ml. of 1:6 sulfuric acid. Then add 1 gram of ammonium sulfate to raise the boiling point. Distil, collecting 10 ml. of steam distillate. In doing so

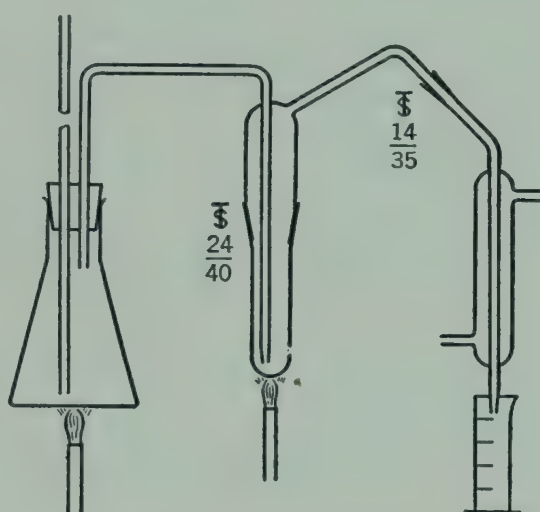

FIG. 20. Distillation apparatus

carry the contents of the tube nearly to dryness. Use the entire distillate as sample.

Urine. Mix 1 ml. of sample, 1 ml. of 2 per cent acetylacetone solution, and 2 drops of 30 per cent sodium hydroxide solution. Heat in boiling water for 10 minutes with an air condenser and cool. Develop with *p*-dimethylaminobenzaldehyde.

Methanol solutions. If streptomycin or dihydrostreptomycin is present with potency less than 1000 micrograms per mg. in methanol, evaporate to dryness carefully. Dissolve the residue in 10 ml. of water and determine by sodium nitroprusside and potassium ferricyanide in alkaline solution.

Procedure—*By ferric ion. Eluate.* Dilute 5 ml. of eluate to about 8 ml., add 0.4 ml. of 10 per cent sodium hydroxide solution, and heat in

a steam bath for 3 minutes. Cool and add 0.4 ml. of 5 per cent ferric ammonium sulfate solution in 1:8 sulfuric acid. Dilute to 10 ml. and read at 540 mμ against a water blank.

Distillate. Over 500 units. Add 1 ml. of 2 per cent ferric chloride hexahydrate in 1:5 hydrochloric acid to the distillate. Read at 550 mμ against a reagent blank.

Under 500 units. Add 1 ml. of phosphotungstic-phosphomolybdic acid (Vol. III, page 116) and after 2 minutes add 3 ml. of 20 per cent sodium carbonate solution. Read at 775 mμ against a reagent blank.

By p-dimethylaminobenzaldehyde. As reagent prepare a 1.33 per cent solution of *p*-dimethylaminobenzaldehyde in 1:1 ethanol-concentrated hydrochloric acid. Mix 2 ml. with 2 ml. of sample solution, previously boiled with acetylacetone, and read against a sample blank.

Streptomycin or dihydrostreptomycin by sodium nitroprusside and potassium ferricyanide. Prepare the reagent by mixing equal volumes of 10 per cent sodium nitroprusside, 10 per cent potassium ferricyanide, and 10 per cent sodium hydroxide solution, in that order. After 15 minutes dilute 1 ml. of this to 100 ml. and shake. Prepare the diluted reagent fresh daily.

To 10 ml. of an aqueous solution containing 200-1000 micrograms of streptomycin or dihydrostreptomycin, add 10 ml. of diluted reagent and let stand for 3 minutes. Read within a half hour against a reagent blank at 490 mμ, using as standard a solution of crystalline dihydrostreptomycin.

Dihydrostreptomycin

Dihydrostreptomycin is oxidized with sodium metaperiodate[46] or periodic acid.[47] Formaldehyde is formed, distilled, and estimated by chromotropic acid.[48] Streptomycin gives 0.6 mole of formaldehyde per mole; dihydrostreptomycin yields 1.6 mole. Each is 0.6 mole high. Periodic and iodic acids interfere but can be quantitatively precipitated by lead acetate.[49] A yellow color in the supernatant layer is not used because it is similar for streptomycin and dihydrostreptomycin. Mannisidostreptomycin gives a pink.

[46] E. A. Garlock, Jr., and D. C. Grove, *J. Clin. Invest.* 28, 843 (1949).

[47] A. Colon, G. E. Herpich, R. G. Johl, J. D. Neuss, and H. A. Frediani, *J. Am. Pharm. Assoc.* 39, 335-8 (1950).

[48] Clark E. Bricker and H. R. Johnson, *Ind. Eng. Chem., Anal. Ed.* 17, 400-2 (1945); William Eisenman and Clark E. Bricker, *Anal. Chem.* 21, 1507-8 (1949).

[49] W. Aubrey Vail and Clark E. Bricker, *Ibid.*, 24, 975-9 (1952).

The difference in absorption of hydrolyzed dihydrostreptomycin at 265 mμ and 380 mμ is a measure of the active content.[50] Both streptomycin and dihydrostreptomycin are determined by sodium nitroprusside and potassium ferricyanide. The method is given under streptomycin.

Procedure—*By periodic acid.* Dilute the sample to contain 4-6 mg. of dihydrostreptomycin or streptomycin, or a mixture, per ml. Mix 2 ml. of this solution with 1 ml. of fresh 10 per cent periodic acid in water and let it stand, stoppered, for 60 $\pm$1 minutes. Add 2 ml. of 15 per cent lead acetate solution, not over 3 days old, to precipitate the excess of reagent. Dilute to 10 ml. and centrifuge thoroughly. Develop 1 ml. of the clear upper layer with 0.5 ml. of 20 per cent solution of chromotropic acid and 6 ml. of concentrated sulfuric acid. Heat in boiling water for 30 $\pm$3 minutes and cool. Dilute to 25 ml. with water and read at 570 mμ against a reagent blank. Dehydrostreptomycin produces 1.02 moles of formaldehyde per mole; streptomycin produces 0.20 mole per mole. If streptomycin exceeds 2 per cent of the mixture, apply a correction from a separate determination of the latter as maltol.

By hydrolysis. Dilute a sample containing 1-3 mg. of dehydrostreptomycin to 3 ml. with water and add 3 ml. of 1:72 sulfuric acid. Attach an air condenser and heat in boiling water for 2 hours. Cool, dilute to 25 ml., and read at 265 mμ and 380 mμ. Apply the difference in the two readings to a calibration curve.

Mannosidostreptomycin (Streptomycin B) and Dihydromannosidostreptomycin

Anthrone gives a characteristic blue-green with carbohydrate in strong sulfuric acid.[51] Only furfural interferes as a test for carbohydrate and the color is the same whether hydrolyzed in the reagent or prior to its addition.[52] Streptomycin and dihydrostreptomycin do not interfere with the use of this for determining streptomycin B and dihydromannosidostreptomycin by its mannose content with this reagent.[53] A suitable standard is mannose.

Another method of determination is by the reaction of the mannose

[50] Dorothy J. Hiscox, *Ibid.* **23**, 923-4 (1951).

[51] Roman Dreywood, *Ind. Eng. Chem., Anal. Ed.* **18**, 499 (1946).

[52] Daniel L. Morris, *Science* **107**, 254-5 (1948).

[53] W. B. Emery and A. D. Walker, *Analyst* **74**, 455-7 (1949); John A. Kowald and Robert B. McCormack, *Anal. Chem.* **21**, 1383-4 (1949).

with carbazole.[54] This also can be applied to get streptomycin by difference from the total by the maltol method. Many reducing sugars give the same reaction with carbazole that mannose does. Nearly all carbohydrates form complexes with a strong maximum at 540 mμ. The yellow color formed by octyl alcohol and pentasol and inorganic ions such as iron, copper, cobalt, and chromium interfere. Amino acids, glycerol, glucosaminic acid, chondrosaminic acid, glucosamine, and N-methyl-l-glucosamine do not interfere. The procedure must be carried out in subdued light since otherwise the carbazole-sulfuric acid solution turns a rather deep green. In adding the sulfuric acid, care must be take not to cause a rise in temperature or the color formed with the carbazole might be quite different from those usually observed.

The pink color of mannisidostreptomycin with periodic acid, with excess reagent removed with lead acetate, is appropriate for differential reading of streptomycin and mannisidostreptomycin.[55] The color development is critical at pH 1.1; at 0.5 or 1.7, no color is formed. Since the calibration curves are not linear over any considerable range, the amount of sample must be more closely controlled than is usual. Reproducibility is then to ±2 per cent.

Methanolysis of streptomycin[56] or of dihydrostreptomycin[57] breaks one of the glucosidic linkages of the molecule to give methyl streptobiosaminide dimethyl acetal or methyl dihydrostreptobiosaminide and strptidine. Methanolysis of mannisidostreptomycin and dihydromannisidostreptomycin gives the same compounds and methyl mannoside[58] A method is therefore based on removal of the ionic products by base exchange, leaving the nonionic methyl mannoside.[59] This is then read as the dinitrophenyl oxazone. A blank prior to methanolysis is necessary to determine any free sugar originally present. Both streptomycin and its hydrolytic products must be removed quantitatively.

Procedure—*By anthrone.* Prepare a fresh 0.2 per cent solution of anthrone in concentrated sulfuric acid diluted to 95 per cent of acid by addition of 100 ml. to 5 ml. of water. As standard use mannose.

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[54] D. Perlman, *J. Biol. Chem.* 179, 1147-54 (1949).

[55] W. Aubrey Vail and Clark E. Bricker, *Anal. Chem.* 24, 975-9 (1952).

[56] N. G. Brink, F. A. Kuehl, Jr., and K. Folkers, *Science* 102, 506-7 (1945).

[57] O. R. Bartz, J. Controulis, H. M. Crooks, Jr., and M. C. Rebstrock, *J. Am. Chem. Soc.* 68, 2163-6 (1946).

[58] H. E. Stavely and J. Fried, *Ibid.* 71, 135-9 (1949).

[59] Joseph Levine, George Seltzer, and William W. Wright, *Anal. Chem.* 25, 671-3 (1953).

Mix 10 ml. of reagent with 5 ml. of sample or standard containing about 0.3 mg. per ml. Let this stand for 15 minutes, at which time it has cooled to room temperature, and read at 650 mμ against a reagent blank. Calculate the mannose from a conversion factor established with the lot of reagent being used. Convert to streptomycin B hydrochloride as containing 21.14 per cent mannose, or as the sulfate 20.28 per cent.

By carbazole. Dilute to 2 ml. a sample containing 0.05-0.15 mg. of mannisidostreptomycin hydrochloride and chill in an ice bath. Add 5 ml. of concentrated sulfuric acid dropwise with shaking to prevent rise in temperature. Add exactly 0.5 ml. of 0.5 per cent solution of carbazole in ethanol. Place in vigorously boiling water for 10 minutes. Cool to room temperature and after 5 minutes make two readings at 540 and 660 mμ against a reagent blank.

Streptomycin B gives a purple-red color under these circumstances and streptomycin A gives a yellow-brown. The colored complex formed by streptomycins A and B give an absorption maximum between 520 and 560 mμ. At 660 mμ the absorption of both complexes is practically identical and the quantity of streptomycin B is calculated as follows:

$$\mathrm{B} = \frac{D_{540}\left(\frac{C_{A1}}{C_A}\right) - D_{660})}{(C_B\left(\frac{C_{A1}}{C_A}\right) - C_{B1}}$$

where

B = amount of streptomycin B in mg.
D_{660} and D_{540} = extinction coefficients
C_A and C_B = Beer's law constants at 540 mμ/mg. of streptomycin A and B
C_{A1} and C_{B1} = constants per mg. of streptomycins A and B at 660 mμ

By periodic acid. Dilute the sample to contain 5 ± 1 mg. of the sulfates of mannisidostreptomycin and streptomycin. Adjust 1 ml. of this sample to 25° and add 1 ml. of fresh 10 per cent aqueous periodic acid solution. After 30 ± 0.5 minutes add 2 ml. of 15 per cent lead acetate solution, not over 3 days old. Dilute to 10 ml. with water and filter. Read at 25° and 415 mμ and 485 mμ against a water blank after 180 ± 1 minutes from the time of adding the lead acetate solution.

For calculation,

$$A = \text{mg. of streptomycin present}$$
$$B = \text{mg. of mannisidostreptomycin present}$$
$$0.147A + 0.093B = D_{415}\ \text{m}\mu$$
$$0.131A + 0.194B = D_{485}\ \text{m}\mu$$

By 2,4-dinitrophenylhydrazine. To a 0.25-gram sample add 5 ml. of a fresh mixture of 3 ml. of concentrated sulfuric acid in 100 ml. of methanol. Reflux for 2 hours in an all-glass apparatus. Cool and dilute to 100 ml. with water. Prepare the chloride form of Amberlite MB3 or equivalent by passing 2 liters of 1:7 hydrochloric acid over a pound of the resin in a column or percolator. Wash with water until the washings are neutral and filter on a Büchner funnel. Draw air through to give a product with about 40 per cent water content.

Prepare a 200 × 25 mm. ion-exchange column with 30 grams of Amberlite MB3, running in water through the lower stopcock and draining to the surface of the resin bed. Pass the methanolyzate at not more than 3 ml. per minute. Discard the first 90 ml., which are diluted by the water in the column, and collect the balance.

As a sample blank pass a solution of 0.25 gram of sample in 100 ml. of water over 30 grams of the chloride form of the resin in the same manner.

As reagent dissolve 25 mg. of dinitrophenylhydrazizne in 5 ml. of concentrated hydrochloric acid by heating and dilute to 100 ml. with water. To 2 ml. of sample effluent add 1 ml. of reagent. Dry, one-third immersed in a steam bath, using a 19 × 150 mm. test tube annealed at 600° since its last use. This keeps the blank value much lower. Heat about 1 hour after the odor of hydrogen chloride is no longer discernible. As alcoholic sodium hydroxide dilute 25 ml. of 8 per cent aqueous solution to 100 ml. with ethanol. After cooling run 2 ml. of alcoholic sodium hydroxide down the side of the tube. Dilute to 25 ml. with 1:1 water-ethanol. Read after 0.5 hour at 556 mμ against a reagent blank. Subtract the value of a sample blank and read in terms of a mannose curve.

For calculation

$$\%\ \text{Mannisido-compound} = \frac{100 \times \text{weight-of-mannose found}}{\text{weight of sample}} \times \frac{100F}{\text{value of aliquot}}$$

$$\text{where } F = \frac{\text{molecular weight of mannisido-compound}}{\text{molecular weight of mannose}}$$

	Values of F
Mannisidostreptomycin sulfate	5.04
Dihydromannisidostreptomycin sulfate	5.05
Mannisidostreptomycin calcium chloride complex	5.40

Aureomycin

When heated with hydrochloric acid, aureomycin develops a yellow color with a maximum absorption at 445 mμ.[60] The color is stable for at least 3 hours. Results agree with the fluorometric method. Buffered to pH 6.5 a maximum fluorescence is reached in 20 minutes; at 100° in 3 minutes. The fluorescence so developed is stable for several hours. Oxidation products do not fluoresce.

Sera, urine, spinal fluid, etc., are chromatographed on silica gel followed by isotonic sodium chloride and ethanol.[61] An alternative is Decalso followed by water and ethanol.[62] Elution is by hot 5 per cent sodium carbonate solution followed by fluorescence reading.

Aureomycin is determined in preparations of high purity simply by making proper dilutions in sulfuric acid solutions and measuring the ultraviolet absorbance at 274 and 350 mμ.[63] The difference in absorbance is proportional to the aureomycin content. The method gives results agreeing with bioassay methods within about ±5 per cent.

Sample—*Ointment.* Dissolve 0.3-1.5 mg. in 10 ml. of ether and extract aureomycin quantitatively with three 5-ml. portions of water. Read in the ultraviolet.

Tablets. Powder 1-5 mg. and shake with two 5-ml. portions of ethanol. Combine the ethanol extracts and dilute with water to 50 ml. Read in the ultraviolet.

Capsules. Combine the contents to give suitable size of sample. Dilute with ethanol for reading in the ultraviolet.

Procedure—*By hydrochloric acid.* Treat 1 ml. of sample solution containing approximately 0.2-1 mg. of aureomycin with 5 ml. of 1:5 hydrochloric acid. Treat a corresponding sample blank by addition of 5 ml. of water. Heat in boiling water for 5 minutes and cool. Add 5 ml.

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[60] Joseph Levine, Edward A. Garlock, Jr., and Henry Fischbach, *J. Am. Pharm. Assoc.* **38**, 473-5 (1949).

[61] John C. Seed and Catherine E. Wilson, *Science* **110**, 707-8 (1949).

[62] Abraham Saltzman, *J. Lab. Clin. Med.* **35**, 123-8 (1950).

[63] Dorothy J. Hiscox, *J. Am. Pharm. Assoc.* **40**, 237-40 (1951).

of 1:5 hydrochloric acid to the sample and dilute each to 50 ml. with water. Read the unknown against the sample blank at 440 mμ.

By fluorescence. As buffer mix 8 parts of 20 per cent dipotassium phosphate solution with 1 part of 20 per cent monopotassium phosphate solution to give a buffer for pH 7.6, or on dilution in use for pH 7.5. Mix 10 ml. of solution containing 0.000025-0.000035 mg. of aureomycin with 2 ml. of buffer. Read the fluorescence at once as a blank with Coleman filters B1 and B2 against 20 mg. of quinine sulfate per liter of 1:360 sulfuric acid, further diluted 5 ml. per liter with the same acid. Heat in a boiling water bath for 5 minutes and cool. Read as before at 15-60 minutes and subtract the blank. The average reading is 2.2 ±0.04 scale unit per 0.00001 mg.

In the ultraviolet. To a 5-mg. aqueous aliquot containing 0.1-0.5 mg. of aureomycin add 5 ml. of 1:17 sulfuric acid. Heat in boiling water for 8 minutes and then cool. Dilute to 25 ml. Read the solution at 274 mμ and 350 mμ. The difference in readings at the two wave lengths is proportional to the aureomycin content.

Erythromycin

When erythromycin is heated with strong acid it is degraded to a yellow compound.[64] Hydrochloric acid can be used but sulfuric is preferred, for one reason because it gives more intense color. The color intensity is about the same for 12-14 *N* acid so that a 1:1 dilution of the sample with 27 *N* acid is used. The heat of dilution is sufficient to develop the color. Impurities in beers are avoided by extraction technics. Degradation products react much like the antibiotic.

Sample—*Solid preparations.* As a 0.1 *M* phosphate buffer for pH 7 dissolve 2.72 grams of monopotassium phosphate and 6.84 grams of dipotassium phosphate trihydrate in water and dilute to 500 ml. Prepare a solution of sample in this buffer to contain about 0.05 mg. per ml.

Beer. As 0.1 *M* carbonate buffer for pH 9.5 dissolve 7.47 grams of potassium bicarbonate and 3.50 grams of anhydrous potassium carbonate in water and dilute to 1 liter. Dilute the beer with this buffer to give a concentration of test substance of about 0.05 mg. per ml. The mycelium need not be filtered out. Shake vigorously 20 ml. with 20 ml. of amyl acetate and centrifuge. The amyl acetate should be previously shaken

[64] Jared H. Ford, George C. Prescott, J. W. Hinman, and E. Louis Caron, *Anal. Chem.* 25, 1195-7 (1953).

with 0.1 volume of 5 per cent potassium bicarbonate solution. Shake 10 ml. of the amyl acetate layer with 10 ml. of 1:160 hydrochloric acid and withdraw the aqueous layer for development under the procedure.

Procedure—To 5 ml. of prepared sample add 5 ml. of sulfuric acid prepared by addition of 75 ml. of concentrated acid, with cooling, to 35 ml. of water. After 30 minutes read at 485 mμ against water.

CHAPTER 14

HEMOGLOBIN AND RELATED COMPOUNDS[1]

The subject is complex. The pigments are combinations of two parts, the heam pigment called hemochromogen and the globin, a protein. Neither is necessarily the same in hemoglobin from different sources. Further in some forms the iron in the haem pigment is reduced; in others not occurring in the body it is oxidized. Nearly all forms are used for determination.

The subject is sufficiently complex so that it becomes necessary to define terminology. The reduced pigment uncombined is hemoglobin, what has sometimes been called ferrohemoglobin to distinguish it from the oxidized forms of the pigment. Reduced hemoglobin is present only in the absence of substances with which it forms loose combination. Further when combined forms are reduced with sodium hydrosulfite or dithionate, the original hemoglobin is not obtained.

Hemoglobin reacts with oxygen to form oxyhemoglobin, $Hb + O \rightleftharpoons HbO$, an equilibrium which readily migrates to the left. The iron in this form is still in the ferrous form. The same holds true of the combination with carbon dioxide. Combined with carbon monoxide it is carbon monoxyhemoglobin, often called carboxyhemoglobin. With cyanide, oxyhemoglobin forms cyanhemoglobin and, with hydrogen sulfide, sulfhemoglobin. All of these are equilibrium products of varying stability.

Oxyhemoglobin is transformed into methemoglobin by many compounds. Combined with cyanide it becomes cyanmethemoglobin corresponding with ferrihemoglobin cyanide. Hematin is formed by oxidation of hemochromogen and is converted by alkali to alkaline hematin. In acid solution hematin forms hetatoporphyrins.[2]

Red cell hemoglobin with a molecular weight of the order of 67,000 is essentially different from muscle hemoglobin of a molecular weight of about 17,000. The red cell hemoglobin has essentially the same value

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[1] See Volume III, Chapter 1, for details of organization, condensation, etc.

[2] A system of designation of reduced forms as hemoglobin and oxidized forms as hemiglobin has been proposed. R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," p. 209. Interscience Publishers, Inc., New York, N. Y. (1949).

whether derived from man, sheep, rat, horse, rabbit, frog, or many birds.

In analytical determination, one may go to fundamentals and determine the iron directly as the most reliable method.[3] Methods may be drastic by oxidation or may liberate iron as by thiolactic acid [4] or potassium persulfate. Values by different methods often differ,[5] but so do standards for the amount present.[6] The following are typical as representing grams per 100 ml.

Dare	= 13.77
Haldane	= 13.80
Oliver	= 15.00
Tallqvist	= 15.80
Von Fleischl-Miescher	= 15.80
Newcomer (Williamson)	= 16.92
Sahli	= 17.20

Because of their diversity, Table 16, gives the percentage equivalents for various amounts of hemoglobin in terms of the different standards. As a convenient mechanism, each form determined is classified under a separate subhead.

Direct Reading

In its elementary form for clinical use, a drop of blood is placed on absorbent paper, allowed to dry, and compared with a color chart.[7] This is no more than a rough estimation.

Since blood is itself colored, the immediate thought would be to read

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[3] Vol. II, pages 296-300; A. D. Marenzi and E. Lida, *Rev. soc. argentina biol.* **14**, 339-46 (1938); R. Belli, A. Boni, and C. Pandolfi, *Diagnostica tec. lab.* (Napoli), *Riv. mensile* **12**, 337-45 (1941); Johan T. Peters, *Southern Med. J.* **40**. 924-6 (1947); E. J. King, M. Gilchrist, et al., *Lancet* **255**, 563-6 (1948).

[4] B. Shorland and E. M. Wall, *New Zealand J. Sci. Tech.* **18**, 93-4 (1936).

[5] Cf. Gregers Sorensen, *Ugeskrift Laeger* **103**, 1453-5 (1941); K. Humperdinck, *Deut. med. Wochenschr.* **69**, 815-16 (1943); E. J. King, Margaret Gilchrist, and Audrey Matheson, *Brit. Med. J.* **1944**, **I**, 250-2; P. Chevallier, A. Fiehrer, and Fr. Saison, *Sang* **16**, 223-8 (1944); E. J. King, M. Gilchrist, I. D. P. Wooton, et al. *Lancet* **253**, 789-92 (1947).

[6] César F. Merino, *Actas y trabojos congr. peruano quím.* **2**, **II**, 336-8 (1943).

[7] Alfred E. Koehler, *J. Biol. Chem.* **58**, 813-30 (1923-4); O. Amme, Brit. Patent 280,551 (1926); Carl A. Hausser and Anthony A. Hausser, U. S. Patent 1,878,847 (1932).

TABLE 16. HEMOGLOBIN IN TERMS OF PERCENTAGE EQUIVALENTS ACCORDING TO VARIOUS STANDARDS

Gms	*Dare*	*Haldane*	*Newcomer Williamson*	*Oliver*	*Sahli*	*Tallqvist*	*Von Fleischl-Miescher*
6	43.6	43.5	35.6	40.0	34.8	38.0	38.0
7	50.9	50.7	41.5	46.7	44.7	44.3	44.3
8	58.1	58.0	47.4	53.3	46.5	50.6	50.6
9	65.4	65.2	53.3	60.0	52.3	56.9	56.9
10	73.6	72.4	59.2	66.7	58.1	63.3	63.3
11	79.9	79.7	65.1	73.3	64.0	69.6	69.6
12	87.1	87.0	71.0	80.0	69.8	76.0	76.0
13	94.4	94.2	76.9	86.6	75.6	82.3	82.3
14	101.7	101.4	82.9	93.3	81.4	88.6	88.6
15	108.9	108.7	88.8	100.0	87.2	95.0	95.0
16	116.2	115.8	94.7	106.7	93.0	101.3	101.3
17	123.5	123.1	100.6	113.3	98.8	107.6	107.6
18	130.7	130.3	106.5	120.0	104.7	113.9	113.9
19	138.0	137.6	112.4	126.7	110.5	120.2	120.2
20	145.3	144.8	118.4	133.3	116.3	126.6	126.6
21	152.5	152.1	124.3	140.0	122.1	132.9	132.9
22	159.8	159.3	130.2	146.7	128.0	139.2	139.2
23	167.1	166.6	136.2	153.3	133.8	145.6	145.6
24	174.3	173.8	142.1	160.0	139.6	151.9	151.9
25	181.6	181.1	148.0	166.7	145.4	158.2	158.2
26	188.8	188.3	153.9	173.3	151.2	164.6	164.6
27	196.1	195.5	159.8	180.0	157.0	170.9	170.9
28	203.3	202.8	165.7	186.7	162.8	177.2	177.2
29	210.6	210.0	171.6	193.3	168.6	183.6	183.6
30	217.9	217.2	177.5	200.0	174.4	189.9	189.9

it, undiluted in a thin layer,[8] or diluted. Although that can be read at 410-420 mμ with some degree of error due to light obstruction by solid particles,[9] the real trick is to read at the wave length of maximum absorption.[10] One absorption maximum of hemoglobin is at 541 mμ and the green line of mercury at 546 can be used. Readings against a screen of known optical density as the standard are accurate to 1 per cent, but unless the corpuscle stroma are removed, their shadow can cause an error of 3 per cent. By dissolving the corpuscles in 0.4 per cent sodium hydroxide, the formation of cathemoglobin rapidly increases the transparency of the solution. This method is applied with the Duboscq colorimeter instead of a photoelectric instrument.[11] Photoelectric readings are accurate to 0.5 per cent.[12]

Agreement with determination of iron depends on clarity obtained by filtration or prolonged centrifuging. Otherwise the normal error is a positive 10 per cent which in certain leukemias reaches 30-40 per cent.[13] With the mercury arc, oxyhemoglobin, reduced hemoglobin, and carbon monoxide-hemoglobin read the same. Methemoglobin can read 30 per cent low. In yellow sodium light methemoglobin reads accurately, but carbon monoxide-hemoglobin or reduced hemoglobin can read 50 per cent low.

Reading in daylight,[14] as with a green filter,[15] is less accurate but much simpler and more practical for routine use. While theoretically filtration to remove leucocytes and erythrocyte debris is indicated, practically thorough centrifuging will serve.

Hemoglobin can be read in the near ultraviolet.[16] The wave lengths for maximum absorption for red cell hemoglobins are hemoglobin 425 mμ (and 541 mμ), carboxyhemoglobin 420 mμ, oxyhemoglobin 414.5 mμ, and methemoglobin 406 mμ. The corresponding values for muscle

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[8] G. A. Harrison, *Lancet* **1938,II,** 621-2.

[9] A. A. Il'ina, Kh. M. Ravikovich, D. L. Rubenshteĭn, and E. V. Shpol'skiĭ, *Compt. rend. acad. sci.* URSS **48**, 325-8 (1945).

[10] A. Dognon, *Compt. rend. soc. biol.* **129**, 467-70 (1938).

[11] A. Dognon and Y. Simonot, *Sang* **16**, 312-14 (1944-45).

[12] E. B. Reeve, *J. Path. Bact.* **56**, 95-107 (1944).

[13] M. Guillot and H. Renault, *Sang* **16**, 243-6, 255-8(1944).

[14] P. Chevallier, A. Fiehrer, Fr. Saison, and J. C. Caine, *Sang* **16**, 249-55 (1944).

[15] Don H. Duffie, *J. Am. Med. Assoc.* **126**, 95-6 (1944); *Am. J. Clin. Path. Tech. Sect.* **9**, 27-9 (1945).

[16] E. M. Jope, "Haemoglobin," pp. 205-19, Interscience Publishers, Inc., New York, N. Y. (1949).

hemoglobins are 435 mμ, 424 mμ, 418 mμ, and 407 mμ. They conform to Beer's law up to 38 per cent.

Spectral absorption in this region is due to the aromatic amino acid part of the pigment; that in the usual visible spectrum to the haem portion. The effects are largely independent. Those due to the haem portion are varied by the combination in which the central iron atom is combined and by other factors. Hydrogen-ion concentration has relatively little effect up to pH 9.

Procedure—*Blood. Micro.*[17] Dilute a 0.2-ml. sample with 0.85 per cent sodium chloride solution containing heparin and centrifuge out the red cells. Hemolyze the cells and read. Alternatively,[18] dilute the sample 1:200. Dilute 0.025 ml. of this to 0.5 ml. with 1:90 ammonium hydroxide and read at 576 mμ. Dilute hemolyzed blood gives a practically clear solution with as low as 0.01-0.1 per cent of ammonia or sodium carbonate present.[19]

As another technic,[20] dilute a 0.02-ml. sample to 2 ml. with 0.1 per cent saponin solution. After 30 minutes for hemolysis, centrifuge to remove stroma and read.

Blood sausage.[21] Heat a 5-gram sample for 2 minutes with 20 ml. of 2 per cent potassium hydroxide in ethanol. Filter, dilute to an appropriate volume with 70 per cent ethanol, and compare with a standard of 0.125 per cent hemoglobin in 70 per cent ethanol as representing an average blood. Alternatively, read in a closely-cut band around 541 mμ.

By Benzidine

By the action of hydrogen peroxide on a mixture of benzidine and diluted blood for a definite time there develops a blue color, which changes to a reddish-brown. The intensity is proportional to the amount of hemoglobin which serves as a catalyst for the oxidation of benzidine

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17 Bacon F. Chow, Lois Hall, B. J. Duffy, and Carl Alper, *J. Lab. Clin. Med.* 33, 1440-6 (1948).

18 R. Belli, A. Boni, and C. Pandolfi, *Diagnostica tec. lab.* (Napoli), *Riv. mensile* **12**, 198-207 (1941).

19 B. Szigeti, *Biochem. J.* **34**, 1460-3 (1940).

20 M. Piette, *Compt. rend. soc. biol.* **139**, 653-6 (1945).

21 R. Gran, *Vorratspflege u. Lebensmittelforsch.* **4**, 62-73 (1941).

by hydrogen peroxide.[22] Since the reagents without blood give substantially no color in 2 hours, there is no reason to believe that direct oxidation of benzidine by hydrogen peroxide occurs. The colored substance is finally dissolved in acetic acid for comparison. This method is useful for measuring the small amounts of hemoglobin present in plasma, urine, or feces.

The peroxide must be prepared by dilution of a 30 per cent grade to avoid stabilizers. The concentration of hydrogen peroxide and reaction time are compensating factors. The development of color is accelerated by temperature and is the same whether in the dark or in the light. Normally errors amount to less than ±1 per cent and are due to mechanical factors.

The colors produced by equivalent amounts of oxy-, met-, carbonyl-, and cyanohemoglobin are the same. Benzidine protects the hemoglobin from acid hydrolysis to hematin when the blood is added to the benzidine solution.[23] A modification [24] uses the pyridine homochromogen.

Procedure—*As blue color. Blood.* Dilute 0.02 ml. of blood to 20 ml. with a solution containing 0.65 per cent of sodium chloride and 0.15 per cent of sodium oxalate. To 1 ml. of this add 15 ml. of a 0.02 per cent solution of benzidine, 3 ml. of 1:400 acetic acid, and 1 ml. of 1 per cent hydrogen peroxide. Mix and cool in ice for 35 minutes. Read at 660 mμ.

Plasma.[25] As reagent dissolve 0.14 gram of benzidine dihydrochloride in 200 ml. of water by heating, add 3.3 ml. of 1:10 acetic acid, and dilute to 500 ml. To 0.4 ml. of plasma and 3.6 ml. of water add 15 ml. of 0.02 per cent solution of reagent and 1 ml. of 1 per cent hydrogen peroxide. Store in an ice bath for 35 minutes and read at 660 mμ.

Alternatively,[26] as reagent dissolve 2 grams of benzidine in 20 ml. of

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[22] Hsien Wu, *J. Biochem. (Japan)* 2, 189-94 (1923); Franklin C. Bing and Reginald W. Baker, *J. Biol. Chem.* **92**, 589-600 (1931); Wm. D. McFarlane and Reginald C. McK. Hamilton, *Biochem. J.* **26**, 1050-60 (1932); T. V. Letonoff, *J. Lab. Clin. Med.* **20**, 66-9 (1934); J. F. Barrett, *Brit. J. Exptl. Path.* **21**, 22-5 (1940); Edmund Berney Flink and Cecil James Watson, *J. Biol. Chem.* **146**, 171-8 (1942); J. Raymond Klein, *Arch. Biochem.* **8**, 421-4 (1945).

[23] Eugenia Sacerdote, *Atti accad. Lincei, Classe sci. fis. mat. nat.* **26**, 116-22 (1937).

[24] J. Garcia-Blanco and S. Grisolia, *Anales inst. med. exptl. Valencia* **3**, 171-3 (1945).

[25] W. C. Karr and F. W. Chornock, *J. Clin. Invest.* **26**, 685-6 (1947).

[26] Morton C. Creditor, *J. Lab. Clin. Med.*, **41**, 307-11 (1953).

glacial acetic acid and dilute to 100 ml. with water. Treat with 1 gram of activated carbon to decolorize, and filter. Mix 2 ml. of reagent with 0.5 ml. of plasma containing less than 0.1 mg. of hemoglobin per ml. Add 1 ml. of a 1:1 mixture of 10-volume hydrogen peroxide and water and mix. After 1 hour at room temperature dilute to 100 ml. with 1:4 acetic acid and let stand for 30 minutes. Read at 490 mμ against a reagent blank.

Sheep feces.[27] This method is also applicable to human feces, but the usual methods for determination of blood in human feces are not applicable to feces of sheep because of the large quantities of coloring matter and plant fiber that are present.

Grind the fresh sample in a mortar until it will pass through a 10-mesh screen. Boil 1 gram with 15 ml. of glacial acetic acid. Centrifuge for 5 minutes and decant. Repeat the extraction 3 times, using 10 ml. of glacial acetic acid, and dilute the decantate to 50 ml. with glacial acetic acid.

Dilute a 1-ml. aliquot to 10 ml. with glacial acetic acid. Add a 1-ml. aliquot of this solution to 2 ml. of 0.02 per cent solution of benzidine and mix. Add 1 ml. of 0.6 per cent hydrogen peroxide solution and stopper. After 2 hours, add 20 ml. of a 20 per cent solution of glacial acetic acid, invert several times, and allow to stand for 8 minutes. Read at 660 mμ. Unless analyzed when fresh, part of the hemoglobin appears to be digested.[28]

As red-brown color. Blood. Add 0.0025 ml. of blood to a 10-ml. volumetric flask. Flush the pipet with water and dilute to 10 ml. Take 2 ml. of 2 per cent benzidine solution in each of 2 tubes. Add 1 ml. of the diluted blood sample to one tube and 1 ml. of diluted blood standard to the other. Mix and add 1 ml. of 1.5 per cent hydrogen peroxide solution to each. After 1 hour add 21 ml. of 1:4 acetic acid to each and mix. Read against a reagent blank.

As Acid Hematin

The oldest and best-known method of estimation of hemoglobin is by conversion at 1:500 dilution to a dispersion of acid hematin.[29] This is then estimated by a natural standard, or liquid or solid artificial stan-

[27] John S. Andrews and H. J. Brooks, *J. Biol. Chem.* 138, 341-51 (1941).

[28] John Southard Andrews and Jose Oliver Gonzales, *J. Lab. Clin. Med.* 27 1212-17 (1942).

[29] H. Sahli, "Lehrbuch der Klinische Untersuchungsmethoden," 5th ed., p. 845, F. Deuticke, Leipsig (1909); K. Bürker, *Arch. ges. Physiol.* 142, 273-90 (1912).

dards, or read photometrically. The pH must be below 4. Breaking the linkage of the two parts is shown by the purple being altered to reddish-brown of the heme pigment. The color takes time to develop and is affected by temperature.[30] It is usual to read this with a special instrument, the hemometer (Figure 21).

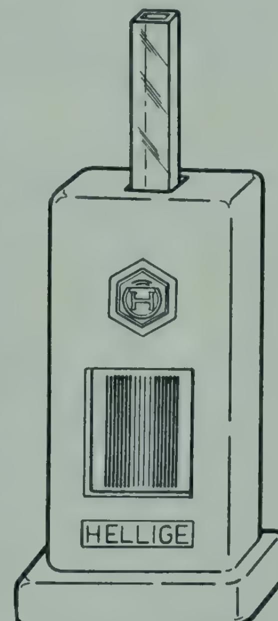

Fig. 21. Sahli hemometer

The colors from whole blood and from red cell suspensions differ.[31] Some types of blood form hematin at a different rate than others, [32] but development at 60° is complete in 15 minutes.[33] At room temperature, approximately 90 per cent of the color develops in 30 minutes and all the color in 180 minutes.[34] The method is more accurate than the carbon monoxide-hemoglobin method.[35] All the hemoglobin compounds including sulfhemoglobin react, although at differing rates.

The color of acid hematin agrees within ±5 per cent with determination of iron.[36] Photometric reading is usually over the entire gray scale with accuracy to ±4 per cent [37] as contrasted with a usual ±10 per cent by this method. Acid hemoglobin from avian blood is best read at 410 mμ.[38] A reproducible stable artificial standard [39] of pH 2.15 at 18°, equivalent to 15.6 ±0.2 grams of hemoglobin per 100 ml., is obtained by boiling 11.61 grams of chrome alum, 13.10 grams of cobalt sulfate, 0.69 gram of potassium bichromate, and 1.8 ml. of 1:35 sulfuric acid in 500 ml. of water for 1 minute, and diluting the cooled solution to a liter.

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30 G. H. Bell and Mary L. McNaught, *Lancet* **1944**, **II**, 784.

31 Georg Barkan and Juta Olesk, *Biochem. Z.* **289**, 251-65 (1937).

32 L. Heilmeyer and I. von Mutius, *Deut. Arch. klin. Med.* **182**, 164-75 (1938).

33 Koziro Matuda, *Japan J. Med. Sci. VIII. Internal med., Pediat. Psychiat.* **5**, *Proc.* 28-9 (1938).

34 Eric Ponder, *J. Biol. Chem.* **144**, 339-42 (1942).

35 Tukayuki Aiki, *Okayama Igakkai Zassi* **51**, 2462-9 (1939); Cf. Einar Bierring, *Nord. Med.* **6**, 953-8 (1940); *Trop. Diseases Bull.* **38**, 348 (1941); Georg Barkan, *Biochem. Z.* **294**, 239-48 (1937).

36 László Urbányi, *Mezögazdasági Kutatások* **15**, 194-9 (1942).

37 Paul L. McLain and George J. Pastorius, *J. Lab. Clin. Med.* **26**, 1054-7 (1941); R. G. Macfarlane, E. J. King, I. D. P. Wooton, and M. Gilchrist, *Lancet* **254**, 282-6 (1948).

38 H. H. Rostorfer, *J. Biol. Chem.* **180**, 901-11 (1949).

39 Q. H. Gibson and D. C. Harrison, *Biochem. J.* **39**, 490-7 (1945).

The color of acid hematin is nearly matched by yellow glasses. The standard in the colorimeter therefore can be replaced by a glass disc and by suitable calibration the instrument will read directly in per cent hemoglobin present as acid hematin.[40] In practice the disc is sometimes incorporated in the plunger of a special instrument. It is necessary to standardize time as well as the dilution. Readings are accurate to ±1 per cent.

Samples—*Human blood.* Dilute 0.05 ml. of blood by delivery under 1:100 hydrochloric acid. Rinse the pipet with the supernatant fluid. Dilute to 25 ml. with 1:100 hydrochloric acid and mix. After 90 minutes read at 635 mμ. The dilution may be varied for anemic bloods. Alternatively,[41] to standardize the degree of color development immerse the tubes, fitted with air condensers, in a 45° bath for 15 minutes, cool in ice, and read at 635 mμ. See other details under procedure.

Blood for Newcomer's method. Mix 0.01 ml. of blood by delivery under 5 ml. of 1:35 hydrochloric acid, thus giving a total volume of 5.01 ml. Read after 25 minutes at room temperature.

Chicken blood.[42] To avoid turbidity in the Newcomer method for chicken blood, dilute 0.02 ml. of blood with 5 ml. of 1:100 ammonium hydroxide solution. Transfer to a tube and add 0.12 ml. of concentrated hydrochloric acid. Shake carefully to aggregate insoluble matter. Read at 635 mμ after 40 minutes, applying other details under procedure. To correct for the increase in volume by the added acid, multiply the result obtained in the usual way by 1.02.

Tissue.[43] Mince a 2-4 gram sample and extract with about 8 ml. of acetone. Centrifuge and decant. Add 3 ml. of a mixture of equal parts of 1:5 hydrochloric acid and glacial acetic acid. Add portions of a 4:1 ether-ethanol mixture until the tissue is white, the final extraction being overnight at 5°. Store the combined extracts at 5° for 12 hours, filter, and dilute to 100 ml. Read the clear ethereal solution of acid hematin at 635 mμ and apply other details under procedure.

Procedure—Note the time after adding the blood to the acid before reading the color and the temperature, and apply the nomogram of

[40] H. S. Newcomer, *J. Biol. Chem.* **37**, 465-96 (1919); *Ibid.* **55**, 569-74 (1923); R. P. Kennedy, *Am. J. Physiol.* 78, 56-63 (1926).

[41] C. A. Ashford, *Brit. Med. J.* **1943**, 575-7; *Analyst* **69**, 55 (1944).

[42] M. O. Schultze and C. A. Elvehjem, *J. Biol. Chem.* **105**, 253-7 (1934).

[43] Albert Greenberg and Dorothy Erickson, *Ibid.* **156**, 679-82 (1944).

Figure 22 as follows. The rate of color development is accelerated by increase of temperature and the total color increases with time. The time before reading should never be less than the first 5 minutes of rapid color development.

With a transparent rule join the time allowed for color development on scale 1 at the right to the temperature on scale 2, the curved one,

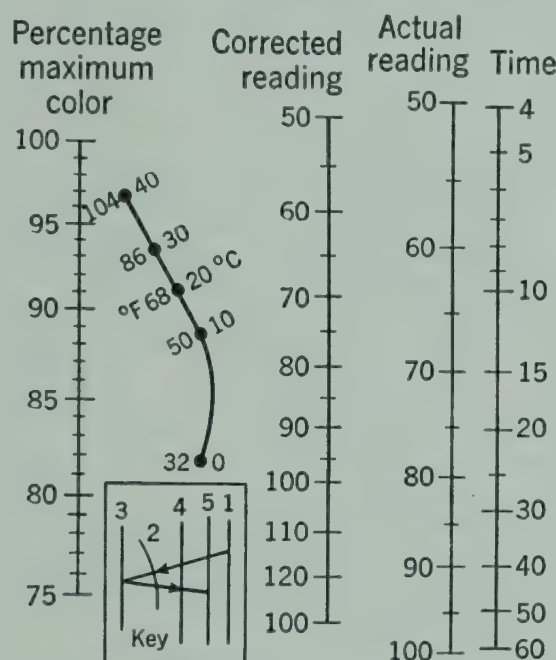

Fig. 22. Nomograph for correction of hemoglobinometer readings for time and temperature [44]

and extend it to scale 3 at the left to give the percentage of the maximum which will have developed in that time at that temperature. Noting that figure, join it to the actual scale reading on scale 5, next to the right scale, which line intersects scale 4 to give the corrected reading. Should the actual reading be less than 50, multiply it by 2 to bring it on the scale and divide the corrected reading by 2.

The key on the nomogram illustrates the application if read at 9 minutes at 20° with an actual reading of 80.

By Newcomer disc. Place the disc under the standard cup of the colorimeter, unless it is part of the plunger. Fill the cup with water and set the plunger at 20 mm. Fill the sample cup with the acid hematin

[44] G. H. Bell and Mary L. McNaught, *Lancet*, **1944**, **II**, 784.

solution. Adjust the sample solution to match the standard. The grams of hemoglobin per 100 ml. of the whole blood are conveniently obtained from Table 17. The figures there given are for No. 3611 yellow filter under the cup, No. 3610 blue filter in the eyepiece, and a filter factor of 0.252. Multiply the value obtained by the correction factor engraved on

Table 17. Conversion Factors for mm. to Grams Hemoglobin per 100 ml. of Whole Blood using Newcomer Disc

mm.	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
5.0	25.25	24.82	24.40	23.98	23.56	23.14	22.72	22.30	21.88	21.46
6.0	21.04	20.74	20.44	20.14	19.84	19.54	19.24	18.94	18.64	18.34
7.0	18.04	17.81	17.59	17.36	17.14	16.91	16.68	16.46	16.23	16.01
8.0	15.78	15.60	15.43	15.25	15.08	14.90	14.73	14.55	14.38	14.20
9.0	14.03	13.89	13.75	13.61	13.47	13.33	13.19	13.05	12.91	12.77
10.0	12.63	12.51	12.40	12.28	12.17	12.05	11.94	11.82	11.71	11.59
11.0	11.48	11.38	11.29	11.19	11.10	11.00	10.90	10.81	10.71	10.62
12.0	10.52	10.44	10.36	10.28	10.20	10.11	10.03	9.95	9.87	9.79
13.0	9.71	9.64	9.57	9.50	9.43	9.36	9.30	9.23	9.16	9.09
14.0	9.02	8.96	8.90	8.84	8.78	8.72	8.66	8.60	8.54	8.48
15.0	8.42	8.37	8.31	8.26	8.21	8.15	8.10	8.05	8.00	7.94
16.0	7.89	7.84	7.80	7.75	7.71	7.66	7.61	7.57	7.52	7.47
17.0	7.43	7.39	7.35	7.30	7.26	7.22	7.18	7.14	7.09	7.05
18.0	7.01	6.97	6.94	6.90	6.86	6.82	6.79	6.75	6.71	6.68
19.0	6.64	6.61	6.57	6.54	6.51	6.47	6.44	6.41	6.38	6.34
20.0	6.31	6.28	6.25	6.22	6.19	6.16	6.13	6.10	6.07	6.04
21.0	6.01	5.98	5.96	5.93	5.90	5.87	5.85	5.82	5.79	5.77
22.0	5.74	5.71	5.69	5.66	5.64	5.61	5.59	5.56	5.54	5.51
23.0	5.49	5.47	5.44	5.42	5.40	5.37	5.35	5.33	5.31	5.28
24.0	5.26	5.24	5.22	5.20	5.18	5.15	5.13	5.11	5.09	5.07
25.0	5.05	5.03	5.01	4.99	4.97	4.95	4.93	4.92	4.90	4.88

Courtesy of Bausch and Lomb Optical Co

the Newcomer disc, which is a standardization factor determined for that disc. If the diluted blood has not stood for 25 minutes, further correct by adding the proper correction figure from Table 18.

Table 18. Correction Factors for Time of Standing in Newcomer Method for Hemoglobin

Minutes since Dilution	*Reading in Grams*										
	25	23	21	19	17	15	13	11	9	7	5
10	0.95	0.89	0.83	0.76	0.70	0.63	0.56	0.50	0.43	0.37	0.30
15	0.65	0.60	0.55	0.51	0.46	0.42	0.38	0.33	0.29	0.24	0.20
20	0.32	0.30	0.28	0.25	0.23	0.21	0.19	0.17	0.14	0.12	0.10
25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

As Cyanhematin

Instead of reading the color of acid hematin this can be converted into cyanhematin, also referred to as the cyanmethematin, before reading.[44] The color is in true solution, rather than colloidal, it develops at once, and follows Beer's law. Addition of ammonium sulfate will often assist in obtaining optical clarity.[45]

Procedure—*Blood.* Draw a 0.02-ml. sample into a capillary pipet and dry the outside of the needle. Deliver under 10 ml. of 1:120 hydrochloric acid. Rinse the pipet with the supernatant liquid. Mix until hemolysis occurs and the color is that of acid hematin. Then add a 1 per cent solution of sodium cyanide drop by drop, exercising precautions because of liberation of hydrocyanic acid. Continue the addition until the color is converted to that of cyanhematin. Dilute to 25 ml. with water and read in the range 600-660 mμ.[46]

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[44] Robert D. Barnard, *J. Lab. Clin. Med.* **17**, 824-5 (1932); D. L. Drabkin, *Am. J. Med. Sci.* **209**, 268-70 (1945); Herman Cohen and Robert D. Barnard, *Am. J. Clin. Path. Tech. Sect.* **10**, 134-7 (1946); Earl J. King and Margaret Gilchrist, *Lancet* **253**, 201-5 (1947).

[45] J. Raymond Klein, *Arch. Biochem.* **8**, 421-4 (1945).

[46] R. Havemann, F. Jung, and B. von Issekutz, Jr. *Biochem. Z.* **301**, 116-24 (1939).

As Alkaline Hematin

The alkaline hematin formed by peroxide and blood in non-acid solution is red-brown.[47] The extinction curve is a linear function of concentration, even with serum present.[48] It estimates abnormal as well as normal hemoglobin.[49] Accuracy is to 0.2 gram per 100 ml.[50] Crystalline hemin in an alkaline buffer is an appropriate standard.[51] There are variations in protein and lipoid forms of hemoglobin which become an important source of error in determinations by the acid hematin, methemoglobin, or cyanhematin forms. Alkaline hematin overcomes these difficulties.

Procedure—*Blood.*[53] As a dilution fluid, stable for two months, use 0.03 per cent of potassium ferricyanide and 0.01 per cent of sodium cyanide in water. Mix 0.03 ml. of blood with 6 ml. of this for reading at 545 mμ after 2 minutes or longer. Alternatively, add 1 ml. of whole blood to 150 ml. of water. Add 4 ml. of 4 per cent potassium ferricyanide solution and after 20 minutes add 1 ml. of 5 per cent potassium cyanide solution. Dilute to 200 ml. and read.

As the Carbon Monoxide-Hemoglobin Complex

Because of the characteristic pigmentation and stability of the carbon monoxide-hemoglobin complex, it is suitable for use in estimation of the total hemoglobin content of blood.[53] While the method presents practical difficulties, it has the advantage of applicability with any species of animal. In a modified form the blood is diluted with very dilute am-

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[47] Hsien Wu, *J. Biochem.* (Japan) 2, 173-80 (1922); D. L. Drabkin and J. H. Austin, *J. Biol. Chem.* 112, 51-65 (1935-6).

[48] M. Laporta and C. Vacca, *Arch. sci. biol.* (Italy), 27, 48-60 (1941); *Boll. soc. ital. biol. sper.* 15, 1094-5 (1940).

[49] J. W. Clegg and E. J. King, *Brit. Med. J.* 1942, II, 329-33; E. J. King, Margaret Gilchrist, and G. E. Delory, *Lancet* 1944, I, 239-43; E. J. King, *Brit. Med. J.* 1947, II, 349-50.

[50] B. L. Horecker, *J. Lab. Clin. Med.* 31, 589-94 (1946).

[51] M. Laporta and C. Vacca, *Boll. soc. ital. biol. sper.* 15, 1094-5 (1940).

[52] Arthur Turner, *Bull. U. S. Army Med. Dept.* 5, 605-7 (1946).

[53] J. Haldane and J. L. Smith, *J. Physiol.* 25, 331-43 (1900); J. Haldane, *Ibid.* 26, 497-504 (1900-1); H. Barcroft, Q. H. Gibson, D. C. Harrison, and J. McMurray, *Clin. Sci.* 5, 145-57 (1945); Kenneth A. Evelyn and Helga Tait Malloy, *J. Biol. Chem.* 126, 655-62 (1938); H. O. Michel and J. S. Harris, *J. Lab. Clin. Med.* 25, 44[illegible]-63 (1940).

nonium hydroxide before saturating with carbon monoxide.[54] The method is also applied to estimation of the fraction of the hemoglobin present which is there as carbon monoxide-hemoglobin. While the original methods are by series of standards, they have been developed photometrically by reading at 430 mμ or 530 mμ.[55] The color develops quickly, is not affected by pH, and is therefore preferable to reading methemoglobin.

Standards—*Haldane method.* Defibrinate about 25 ml. of normal human blood by shaking with glass beads. Determine the hemoglobin content of the defibrinated blood by one of the standard methods. From the determination calculate a dilution to 5 per cent of normal. Thus if the blood showed 85 per cent of normal hemoglobin, 10 ml. would be diluted to 170 ml.

Prepare water saturated with carbon monoxide by passing illuminating gas through it for 1 hour. Use this for dilution in preparing the diluted blood. The color will be the characteristic cherry-red of the carbon monoxide-hemoglobin complex. Cover with a layer of paraffin oil and let stand for globulin to precipitate. When the globulin has separated, decant or centrifuge. Pass illuminating gas slowly through the globulin-free diluted blood, minimizing evaporation with a layer of paraffin oil.

Prepare a series of 20 standards from this diluted blood, using 1 to 20 ml. of the carbon monoxide-hemoglobin solution, and dilute each to 20 ml. with water saturated with carbon monoxide. The tubes should be about half full and, if they are to be sealed in the flame, should have a constriction for that purpoes. Put the tubes in a large desiccator through which illuminating gas is passing. For open tubes, about 3 hours will be required to displace the air over the standards; for tubes with constrictions, a period of about 24 hours is preferable. If the gas from the desiccator is burned, a change in appearance of the flame will indicate the complete displacement of oxygen. Remove the tubes from the desiccator with a finger over the top opening of each and fuse at once; or stopper in the desiccator, remove, and seal by paraffin. Store in the dark when not in use.

Palmer method. Determine the oxygen capacity of blood by one of the recognized methods. Dilute with 1:250 ammonium hydroxide to

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[54] Walter W. Palmer, *J. Biol. Chem.* **33**, 119-26 (1918).

[55] Karl G. Paul and Hugo Theorell, *Acta Physiol. Scand.* **4**, 285-92 (1942).

obtain a 20 per cent dilution of normal blood, that is, one equivalent after dilution to an oxygen capacity of 3.7 per cent. Add a drop of caprylic alcohol to prevent foaming and bubble illuminating gas through this for 10 minutes. Stopper immediately on removing the gas tube and keep cool. This keeps for at least a year.

As a working standard dilute 20 ml. of this stock solution to 100 ml. with 1:250 ammonium hydroxide. Saturate with illuminating gas to give a standard equivalent to a 1 per cent solution of normal blood. This keeps 2-3 weeks when properly protected. On deterioration it shows a brownish tinge.

Procedure—*Total hemoglobin, Haldane method.* Dilute 0.1 ml. of blood to 10 ml. in the pipet with distilled water saturated with carbon monoxide. Discharge into a tube like those used for the standards and add 10 ml. more of water saturated with carbon monoxide. Stopper and observe that the color appears. If it does not, pass in illuminating gas. Globulin precipitates and must be allowed to settle or be centrifuged. Compare with the series of standards, which will read to an accuracy of ±5 per cent. If the sample is darker than the 100 per cent sample, a hyperchrome, dilute the sample further with carbon monoxide solution before examining it. A green glass will improve the accuracy of reading of the darker samples.

Palmer method. Pipet 0.05 ml. of blood sample into 5 ml. of 1:250 ammonium hydroxide solution. Rinse the pipet, mix, and bubble illuminating gas through the solution for 30 seconds. This must be done within 1 hour after mixing. Compare by balancing against a standard equivalent to a similar dilution of normal blood.

As Hemochromogens

When hemoglobin is reduced in alkaline solution by sodium hydrosulfite, $Na_2S_2O_4$, a cherry-red color of complex hemochromogens is obtained,[56] rather than simply the violet of hemoglobin. The color is much like that of carbon monoxide-hemoglobin. The oxyhemoglobin in hemolyzed blood is also completely reduced under these conditions.[57] Degradation is shown by the fact that on oxidation with air and reduction

[56] Anna Dénes, *Biochem. Z.* **255**, 378-86 (1932); Bele Deutsch, *Ibid.* **274**, 299-304 (1934).

[57] H. A. J. Pieters and W. J. Hanssen, *Rec. trav. chim.* **67**, 782-8 (1948).

again the value is not the same.[58] The color of the reduced hemoglobin protected from air is very stable.[59]

Procedure—*Blood.*[60] Mix 0.5 ml. of blood with 1:150 ammonium hydroxide. Add a few crystals of sodium hydrosulfite and dilute to 10 ml. with the ammonium hydroxide. Read at 550 mμ against a reagent blank.

As the Pyridine Compound

A pyridine hemochromogen is formed by reduction with sodium hydrosulfite in alkaline solution in the presence of pyridine.[61] The absorption of the pyridine hemochromogen is about one-third more intense than that of oxyhemoglobin. Excess pyridine is required to complete the reaction. The maximum absorption for normal blood is at 564 mμ, but for dried and reconstituted blood it is at 550 mμ.

By saturation of the solution with carbon monoxide before forming the chromogen, greater accuracy is obtained and possible oxidation by decomposition products of hydrosulfite is avoided.[62] Then it measures all except choleglobin. By reading at 630 mμ the latter is included. The band has been reported at variable wave lengths.[63]

A modified pyridine reagent contains in one part 9 ml. of pyridine, 1 gram of resorcinol, and 5 grams of aminopyrine per 100 ml. of ethanol. The other part is 8 per cent acetic acid in ethanol. Mixing of 0.5 ml. of sample, 2 ml. of the first reagent, 1.5 ml. of the second reagent, and 1 ml. of 0.6 per cent hydrogen peroxide with filtration after 45 minutes and dilution with an equal volume of water gives a color read at 535 mμ[64]

Sample—*Meat scraps or tankage.* Wash a 1-gram sample by soaking with ether for 10 minutes, centrifuge, and repeat twice to complete

[58] R. Havemann, *Klin. Wochschr.* **20**, 362-3 (1941).

[59] K. Bürker, *Verhandl. deut. Ges. inn. Med.* **52**, 351-2 (1940).

[60] Claude Liebecq, *Compt. rend. soc. biol.* **140**, 570-1 (1946).

[61] D. L. Drabkin and J. H. Austin, *J. Biol. Chem.* **112**, 89-103 (1935); Claude Rimington, *Brit. Med. J.* **1942**, I, 177-8; Edmund B. Flink and Cecil J. Watson, *J. Biol. Chem.* **146**, 171-8 (1942); Raymond Reiser and G. S. Fraps, *Ind. Eng. Chem., Anal. Ed.* **14**, 851-3 (1942); A. D. Marenzi and C. E. Cardini, *Anales farm. bioquim.* (Buenos Aires) **15**, 103-7 (1944); Raymond Reiser, *Anal. Chem.* **19**, 114-18 (1947).

[62] R. Lemberg, J. W. Legge, and W. H. Lockwood, *Biochem. J.* **35**, 339-52 (1941).

[63] David L. Drabkin, *J. Biol. Chem.* **140**, 373-85, 387-96 (1941).

[64] R. Ardry and J. Storck, *Ann. pharm. franç.* **9**, 171-5 (1951); *Ann. biol. clin.* (Paris) **9**, 197-200 (1951).

removal of fatty material. Add 100 ml. of a solution containing 55 ml. of ethanol, 5 ml. of pyridine, 5 grams of sodium hydroxide, and water to dilute to volume. Reflux for 30 minutes in an all-glass apparatus and cool. Centrifuge and separate the clear upper layer. Filter for the use of aliquots.

Commercial dried blood. Weigh out a 0.1-gram sample and proceed as above, starting at "Add 100 ml. of . . ."

Fresh blood. Use a sample which will give about 0.1 gram of solids and proceed as above, starting at "Add 100 ml. of . . .."

Plasma hemoglobin content. Withdraw blood without venous stasis into a clean syringe rinsed in physiological saline solution and empty into a known volume of 3 per cent sodium citrate solution. In this way, artificially induced hemolysis is avoided. Centrifuge immediately and remove the plasma. One ml. is a normal sample for development.

Feces. Extract a 10-gram sample with ether or acetone to remove fatty matter. Acidify by adition of 2 ml. of 1:5 hydrochloric acid and 1 ml. of glacial acetic acid. Extract with 4 successive 25-ml. portions of 1:4 ethanol-ether. Decant the extract, filter, and wash the extract with water. Make the ether extract alkaline by extraction with 1:9 ammonium hydroxide. The hemoglobin pigments are extracted into this in three extractions. Dilute to an appropriate exact volume. A usual aliquot from 25-100 ml. dilution is 2 ml.

Urine hemoglobin. Acidify 10 ml. with 5 ml. of 1:5 hydrochloric acid. Extract with four 25-ml. portions of 1:4 ethanol-ether. Break emulsions with a few ml. of ethanol. Wash the ether extract once with water and proceed as for feces, starting at "Make the ether extract alkaline . . .."

Plasma hemoglobin. Centrifuge unhemolyzed blood containing about 3 per cent of sodium citrate to separate the plasma.

Procedure—*Slaughter-house samples.* To 10 ml. of the sample solution containing 5-15 mg. per ml., in which the pyridine is already present, add a few mg. of sodium hydrosulfite. Read the sample solution in which the complex is present against this reduced hemoglobin as blank. Use 564 mμ unless the blood has been dried in which case use 550 mμ.

Biological samples. These do not already contain the pyridine. To a sample containing 0.06-3 mg. of hemoglobin, add 2 ml. of pyridine, 2 ml. of fresh 2 per cent sodium hydrosulfite solution, and enough 1:9 ammonia to dilute to 10 ml. Read at 550 mμ against a blank consisting of a similar tube bleached with hydrogen peroxide by replacing 1 ml. of

ammonium hydroxide with 3 per cent hydrogen peroxide, allowing 5 minutes for decolorization.

Myoglobin

Both hemoglobin and myoglobin give absorption in the range 400-450 mμ.[65] Differential readings at 580 mμ before and after alkaline treatment are suitable for estimation of myoglobin,[66] the alkalinity being sufficient to destroy the methemoglobin.[67] Alternatively use the reduced pyridine hemochromogens in 0.4 per cent sodium hydroxide solution for the second determination.[68]

Sample—*Tissue.* Cut 0.5 gram into small pieces, freeze, and grind with dry ice until homogeneous. Homogenize with 3 ml. of acetate buffer for pH 4.5. Centrifuge, decant, and extract twice more. Combine the extracts and dilute to a known volume. An aliquot of this extract is suitable for determining hemoglobin and myoglobin by development of the pyridine complex. It is also suitable for reading as carbon monoxide-hemoglobin.

Procedure—*By difference.* Adjust the pH of the solution to 9.8. Read at 577-9 mμ, as with filtered rays of a mercury lamp. Add 30 per cent sodium hydroxide solution dropwise to raise the pH to 12 and read again. The second color is metmyoglobin, the methemoglobin having been destroyed by the akali. The difference between the two represents the total hemoglobin.

By multiple readings. To 6 ml. of prepared sample add 25 mg. of solid disodium phosphate dodecahydrate per ml. Determine the hemochromogens as the pyridine complex in a 1-ml. sample. Saturate the remaining 5 ml. with carbon monoxide and read at 568 mμ, 575.7 mμ, and 583.8 mμ. The instrument used must be suitable for reproduction of the wave length within a fraction of a mμ.

$$\text{Total haem pigments} = 1.416 \times A_{575.7}$$
$$\text{Hemoglobin} = 2.2123\ (A_{568} - A_{583.8})$$
$$\text{Myoglobin} = \text{Total} - \text{hemoglobin}$$

The total pigment by the two methods must check.

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65 Margit Benzák, *Acta Chem. Scand.* 2, 333-42 (1948).

66 A. R. Fanelli and L. Guilotto, *Boll. soc. ital. biol. sper.* 24, 506-8 (1948).

67 A. Rossi Fanelli, *Bull. soc. chim. biol.* 31, 457-60 (1949).

68 Hugo Theorell and Christian de Duve, *Arch. Biochem.* 12, 113-24 (1947); Christian de Duve, *Acta Chem. Scand.* 2, 264-89 (1948); E. M. Jope, ''Haemoglobin,'' p. 125-8, Interscience Publishers, Inc., New York, N. Y. (1949).

Oxyhemoglobin

The most important form of hemoglobin, oxyhemoglobin, can be used either for determination of the hemoglobin content or the degree of saturation of the blood with oxygen. It has been read by various means, but the most accurate one is spectophotometric.[69] Practically only hemoglobin and oxyhemoglobin are present in normal blood. A special cuvet with a depth of only 0.007 cm. is used for such reading. Temperature has only a negligible effect on the percentage of oxyhemoglobin between 27° and 37°.

The degree of saturation of blood with oxygen is obtained by matching the color of the blood diluted in the absence of oxygen. Such a match can be obtained only with a special colorimeter having 2 concentric cups on the sample side.[70] The color of blood partially saturated with oxygen is a combination of the yellowish-red of oxyhemoglobin and the violet of hemoglobin. At 536 mμ, the absorption by hemoglobin is about 3 times as great as of oxyhemoglobin.[71] Knowing the value for total hemoglobin in all forms, that for oxyhemoglobin can be calculated.

Another technic is to add the blood sample to an oxygen-free solution of 0.24 per cent ammonia and 0.5 per cent saponin for hemolysis. After reading, the sample is shaken with air to complete conversion to oxyhemoglobin and read again, the original degree of saturation being calculated from the difference.[72] Another basis for the determination is to reduce the sample with hydrosulfite after reading, thus removing all oxyhemoglobin. As much as 13 per cent of the hemoglobin may be inactive so far as formation of oxyhemoglobin is concerned.[73]

Oxyhemoglobin is conveniently read at 510-550 mμ against an artificial standard.[74] An oxygen pressure of about one atmosphere is needed during handling.[75] At 520 mμ methemoglobin and sulfhemoglobin have

[69] David L. Drabkin and Carl F. Schmidt, *J. Biol. Chem.* **157**, 69-83 (1945).

[70] J. Holló and St. Weiss, *Biochem. Z.* **185**, 373-8 (1927).

[71] J. H. P. Jonxis, *Nederland. Tijdschr. Geneeskunde*, **83**, I, 876-80 (1939); Cf. Kenneth A. Evelyn and Helga Tait Malloy, *J. Biol. Chem.* **126**, 655-62 (1938).

[72] Béle Issekutz, Jr. *Math. naturw. Anz. ungar. Akad. Wiss.* **60**, 217-23 (1941); H. A. J. Pieters and W. J. Hanssen, *Rec. trav. chim.* **67**, 782-8 (1948).

[73] Esther Ammundsen and Mogens Trier, *Acta Med. Scand.* **101**, 451-60 (1939); *Arch. Exptl. Path. Pharmakol.* **197**, 332-7 (1941).

[74] Henry F. Holden, *Australian J. Exptl. Biol. Med. Sci.* **21**, 169-70 (1943).

[75] Cesco Toffoli, *Ann. chim. applicata* **38**, 444-8 (1948).

the same absorption as oxyhemoglobin.[76] Each gram of hemoglobin saturated with oxygen binds 1.34 ml. of oxygen.[77]

The usual case is measurement of the difference in oxygen content between venous and arterial blood simultaneously in the same individual. Thus in a simplified form it is assumed that two well-hemolyzed samples of blood drawn nearly simultaneously from the same individual will have substantially the same total hemoglobin content, inactive hemoglobin, turbidity, and content of other light-absorbing material.[78] Consequently, the difference in optical density of the two samples at an appropriate wave length is proportional to the difference in the oxyhemoglobin concentration of the two samples. Degree of saturation of blood samples is measured by the difference between the sample and either reduced or oxygenated hemoglobin.

Sources of error are failure to obtain satisfactory hemolysis and inaccuracy in mixing blood and saponin. Blood and saponin must be mixed thoroughly immediately after being brought together or else a protein precipitate will form at the interface between the two solutions. Cloudiness will develop in 5 minutes. Readings must be taken within 30 minutes after hemolysis.

Unneutralized saponin yields blood saponin mixtures of pH range 6.2-6.5 whose color is not stable. For bloods with a hemoglobin content of less than 8 grams per hundred ml., use neutralized 15 per cent saponin solution. Blood samples with a normal hemoglobin content will give the same readings with either neutralized or unneutralized saponin. Old solutions of saponin give irregular hemolysis.

Procedure—*Blood.* Draw 4-5 ml. of blood into an oiled 10-ml. Luer-Lok syringe, and cap with a soldered needle hub. Have enough heparin solution in the syringe to fill the dead space. Note the volume of sample, add some mercury to aid mixing of the blood and heparin, and store the syringe in ice water.

Hemolyze the sample by adding 30 per cent saponin solution equal to one-fifth the volume of the blood sample. Shake immediately and vigorously. Transfer to a cuvet through a No. 19 needle attached to the

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76 G. H. Bell, J. W. Chambers, and M. B. R. Waddell, *Biochem. J.* **39**, 60-3 (1945).

77 I. A. Oïvin, *Lab. Prakt.* (USSR) **16**, No. 5, 14-15 (1941).

78 J. B. Hickam and R. Frayser, *J. Biol. Chem.* **180**, 457-65 (1945); B. Issekutz, Jr., G. Hetényi, Jr. and I. Feuer, *J. Physiol.* **108**, 9-11 (1949).

syringe. Bend the needle at right angles to avoid injecting mercury from the bottom of the syringe and have it long enough to reach the bottom of the cuvet. Immediately place a layer of mineral oil over the blood. Read the density of the sample at 660 mμ. If absolute values are to be reported, another sample, saturated with air, is run at the same time.

Calculations—The difference in oxygen content between the saturated sample and any other sample is the oxygen difference in volume per cent = 7.76 × optical density difference + 0.36.

Arteriovenous differences are determined by subtracting saturation-arterial difference from saturation-venous difference. Or they can be determined directly by measuring the density of the venous sample against that of the arterial sample and multiplying the optical density difference by 7.76.

Absolute oxygen content can be estimated on the basis of total hemoglobin content and difference in oxygen content between the sample and the saturated blood. Total hemoglobin must first be determined by an appropriate method. Development as cyanhemoglobin is usually recommended for this.

CHAPTER 15

NATURAL PIGMENTS[1]

For the present purpose pigments are large molecules other than hemoglobin which are relatively insoluble in water or reasonably reactive solvents. Thus they are molecules which are inherently of such a size as to have color without the necessity of building it up by additions to the molecule. A typical example is bilirubin as contrasted with the many simple molecules which are coupled with a large molecule to give a color body. Some materials of unknown structure are assumed from this to fit most properly with pigments, largely because of lack of other place to classify them.

Chlorophylls

Chlorophyll exists in *a* and *b* forms of which chlorophyll *a* is normally 68-72 per cent of the total. A major problem is the standard since chlorophyll is degraded on drying. One of the first products of degradation is pheophytin which absorbs near 510 mμ as compared with 660 mμ for chlorophylls.

While the associated carotene and xanthophylls can be separated, washing out xanthophylls with methanol removes chlorophyll *b* because it is more soluble in methanol than chlorophyll *a*. Therefore it is preferable to read chlorophylls by an absolute method[2] rather than against standards[3] or Lovibond glasses.[4]

The maximum absorption of the chlorophylls *a* and *b* in the red visual spectrum is independent of the presence of flavones, xanthopylls, and carotenoids.[5] They are conveniently read in 85 per cent acetone.[6] The red

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[1] See Volume III, Chapter 1, for details of organization, condensation, etc.

[2] C. L. Comar and F. P. Zscheile, *Plant Physiol.* **16**, 651-3 (1941); *Ibid.* **17**, 198-209 (1942); C. L. Comar, *Ind. Eng. Chem., Anal. Ed.* **14**, 877-9 (1942); Erwin J. Berme, Dorothy I. Rose and C. L. Comar, *J. Assoc. Official Agr. Chem.* **27**, 517-26 (1944).

[3] G. Mackinney, *J. Biol. Chem.* **132**, 91-109 (1940); F. P. Zscheile, *Botan. Rev.* **7**, 587-648 (1941); F. P. Zscheile and C. L. Comar, *Botan. Gaz.* **102**, 463-81 (1941).

[4] G. S. Fawcett, *Chemistry and Industry*, **1939**, 362.

[5] H. G. Petering, W. Wolman, and R. P. Hibbard, *Ind. Eng. Chem., Anal. Ed.* **12**, 148-51 (1940); R. B. Griffith and R. N. Jeffrey, *Ibid.* **16**, 438-40 (1944).

[6] Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, 7th Ed., 112-15, Association of Official Agricultural Chemists, Washington D. C. (1950).

fluorescence of chlorophyll *a* is used for its estimation [7] down to 0.001 mg. per liter. It is very sensitive and does not require the detailed purification for other methods. A small amount of turbidity does not interfere and it is rapid. Both the absorption [8] and fluorescent [9] spectra differ for chlorophyll *a* and chlorophyll *b*. The theoretical error in reading chlorophyll *a* by fluorescence due to possible presence of chlorophyll *b* does not exceed 4 per cent.

When sorption methods are applied, carotene can be sorbed on sugar.[10] Ether solutions of chlorophyll do not deteriorate during 3 months of storage in dry ice. Carotene and xanthophylls can be read on the same solution. Frequently the material is saponified and the xanthophyll separated by partition between methanol and petroleum ether. Some carotenoids remain in the petroleum ether [11] but do not interfere.[12]

Samples—*Total chlorophyll in plants.* Cut finely with shears and mix. Dry and grind in a mill. Transfer 5 grams to a mortar with 0.1 gram of calcium carbonate or sodium carbonate. Macerate with the pestle, grind with a small amount of sand, and add small portions of 85 per cent acetone, grinding until well disintegrated. Drain on a funnel with suction and wash the residue with 85 per cent acetone. Grind the residue with more 85 per cent acetone and filter and wash as before. Repeat until the residue is colorless and the extracts show no green. Make the last extraction with absolute acetone and add water to the filtrate to cut back to 85 per cent. Dilute the filtered extract to a known volume and read an aliquot. This extract is also suitable for estimation of mixed carotenes (Vol. III, page 37).

Total chlorophyll and a- and b-chlorophyll in plants. Prepare as before to ". . . wash the residue with 85 per cent acetone." Use ether to remove the last traces of pigment if possible. If necessary reextract. Dilute the filtrates to a known volume with 85 per cent acetone. Mix 50 ml. of ether with 25 ml. of sample in a separatory funnel. Add water carefully until all fat-soluble pigments are in the ether layer. Discard

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[7] F. Kavanagh, *Ind. Eng. Chem., Anal. Ed.* **13**, 108-11 (1941); C. L. Comar, *Ibid.* **14**, 877-9 (1942); Richard H. Goodwin, *Ibid.* **19**, 789-94 (1947).

[8] D. G. Harris and F. P. Zscheile, *Bot. Gaz.* **104**, 515-27 (1943); F. P. Zscheile and C. L. Comar, *Ibid.* **102**, 463-81 (1941).

[9] F. P. Zscheile and D. G. Harris, *J. Phys. Chem.* **47**, 623-37 (1943).

[10] Harold H. Haskin, *J. Biol. Chem.* **144**, 149-60 (1942).

[11] M. E. Wall and E. G. Kelley, *Ind. Eng. Chem., Anal. Ed.* **15**, 18-20 (1943).

[12] D. I. Sapozhnikov, *Doklady Akad. Nauk.* (USSR) **60**, 1361-2 (1948).

the water layer. Place the funnel containing the ether layer over an open tube of about 20-mm. diameter drawn to a fine jet at the bottom. Place a second separatory funnel containing about 100 ml. of water below that. Allow the ether to run through the open tube and rise in small droplets through the water. Rinse the upper separatory funnel and tube with ether. Replace the upper separatory funnel as the lower one containing about 100 ml. of water. Discard the water from the former lower funnel and place it above the tube. Then repeat the washing process. Repeat 5-10 times until all acetone is removed and dilute the ether to 100 ml. for reading.

Procedure—*Total chlorophyll.* Read at 660 mμ against 85 per cent acetone.

Total chlorophyll and a- and b-chlorophylls. Dry an aliquot of ether solution with sodium sulfate until optically clear. Dilute an aliquot to read near 0.6 at 660 mμ. Read against ether at 1 mμ intervals over the range 658-665 mμ. Calculate $\log_{10} I_o/I$ [13] for each wave length. Redetermine if the value at 660 mμ is not the highest. Calculate as follows from values read at 642.5 mμ and 660 mμ.

$$\text{Total chlorophyll} = 7.12 \log_{10} I_o/I \,(\text{at } 660 \text{ m}\mu) + 16.8 \log_{10} I_o/I \,(\text{at } 642.5 \text{ m}\mu)$$

$$\text{Chlorophyll } a = 9.93 \log_{10} I_o/I \,(\text{at } 660 \text{ m}\mu) - 0.777 \log_{10} I_o/I \,(642.5 \text{ m}\mu)$$

$$\text{Chlorophyll } b = 17.6 \log_{10} I_o/I \,(\text{at } 642.5 \text{ m}\mu) - 2.81 \log_{10} I_o/I \,(\text{at } 660 \text{ m}\mu)$$

Chlorophyll a. Fluorometric. Read the sample at 404.7 mμ against 0.1 mg. of quinine sulfate per liter in 1:360 sulfuric acid.

Xanthophyll, Lutein

Since xanthophyll comes out with carotenes and chlorophylls, the sample can be separated to give all three.[14]

Sample—*Leaf tissue.* Macerate 10 grams of sample with a few mg. of calcium carbonate and 125 ml. of acetone in a blender. A glass baffle suspended one-third of the way down from the cover prevents deposition on the cover from which quantitative recovery is difficult. Wash down with acetone once during the 5-minute extraction. Filter on a Büchner funnel and wash the macerate well with acetone. Dilute the filtrate to

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[13] Equivalent to $\log_{10} I_1/I_2$, Vol. I, page 13.

[14] References to xanthophyll are included with chlorophyll.

a known volume and take a 100 ml aliquot. Mix with 50 ml. of ether and add 100 ml. of water. Separate the aqueous acetone layer. Wash this twice with 10-ml. portions of ether to remove xanthophyll and add these to the ether layer. Wash the ether layer by dripping 300 ml. of water through it. Chill the ether layer to 7° to decrease water solubility, withdraw the water, and dry the ether in the refrigerator with anhydrous sodium sulfate. After an hour, filter through cotton, wash the filter with ether, and dilute to 50 ml. at 7°.

Alternatively, for a technic without chromatographing, grind a known weight of plant material with about 3 times its weight of anhydrous sodium sulfate. Extract with hexane and dry the residue which contains the chlorophyll and xanthophyll. Extract this powder with 1:3 ethanol-acetone. This contains the chlorophyll and xanthophyll. Read the chlorophyll as described under the preceding procedure. Add 1 gram of potassium hydroxide and shake mechanically for 15 minutes. Add 6 ml. of light hexane and shake. Add 2 ml. of water and shake. On separation the chlorophyll is in the lower layer, whereas the xanthophyll is in the upper layer. Withdraw the lower layer and extract the hexane with 2, 2, and 2 ml. of water. Read the xanthophyll in the hexane layer as described under procedure.

Procedure—*Carotene.* Sorb other pigments from the carotene by passing 5 ml. of the ether solution through a 15 × 2.5 cm. column containing 6 grams of 5 parts of magnesium oxide and 3 parts of Hyflo Super-Cel at 5 cm. The prepared column is wet with petroleum ether. Wash the carotene through the column with 20 ml. of ether. Wash deposited carotene from the tip of the column. The carotene should have passed completely and the xanthophyll not started to come out of the tap. Dilute the solution to 25 ml. with ether and read (Vol. III, page 37).

Xanthophyll. After the carotenes are eluted, remove the xanthophylls by similar treatment with absolute ethanol, taking care to avoid elution of green pigments. Dilute the xanthophyll fraction to 25 ml. with absolute ethanol and read at 442 mμ. Standardization must be against mixed xanthophylls from a similar source.

Anthocyanins

This important type of pigment as present in strawberries fades and secondary brown pigments appear.[15] Data have been obtained with

[15] Ernest Sondheimer and Z. I. Kertesz, *Anal. Chem.* 245-8 (1948).

Lovibond glasses in terms of decrease of the red-yellow absorption.[16] There is a direct relationship between the pH and the light absorption.[17] Thus the optical density of fresh strawberry juice at 500 mμ more than doubles by lowering the pH from 3.5 to 2. The same is true of pure anthocyanin chloride from strawberries. In neutral or nearly neutral solutions the anthocyanin is probably in the free state but is amphoteric and forms oxonium salts with mineral or organic acids. Recoveries are within 7.1 per cent, but deviations are largely due to the successive operations required. Ethanol shifts the color upward. As little as 2 ppm. of iron alters the color.[18] Temperatures of 18-34° do not affect the results. Solutions stored at 0° do not change for 24 hours.

Sample—*Strawberries, fresh or stored, and preserves.* As buffer dissolve 21 grams of citric acid in 200 ml. of 4 per cent sodium hydroxide. Mix 52.5 ml. of 1:110 hydrochloric acid with 47.5 ml. of this solution as a buffer for pH 3.4. Homogenize an 80-gram sample for 0.5 minute with the 100 ml. of buffer. Centrifuge to separate the bulk of the solids and filter without filter medium other than paper. If difficulty is encountered in getting clear filtrates from fresh strawberries, add about 25 mg. of Pectinol 10M (Rohm and Haas) while blending. The same result is obtained by freezing for several hours. Dilute a portion of the filtrate to give a proper color density for reading. If the pH is not 3.40 ± 0.05, repeat the dilution adding small amounts of citric acid or sodium citrate during dilution.

Dilute another portion of the extract with addition of 1:1 hydrochloric acid to get a final pH of 2.00 ± 0.05. Let it stand for an hour for full color development.

Strawberry juice. Mix 80 grams with 100 ml. of the buffer and proceed as for whole berries.

Procedure—*Against Congo red.* Dissolve 0.0200 gram of Congo red in 0.053 per cent sodium carbonate solution and obtain the transmittance at 500 mμ at various dilutions with 0.053 per cent sodium carbonate solution.

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[16] H. G. Beattie, K. A. Wheeler, and Carl S. Pederson, *Food Research* **8**, 395-404 (1943); H. H. Thompson, S. R. Cecil, and J. W. Woodroff, *Food Industries* **18**, 1341, 1510 (1946).

[17] Edith P. Smith, *Protoplasma* **18**, 112-25 (1933); Heinrich Fincke, *Z. Untersuch. Lebensm.* **82**, 209-15 (1941).

[18] J. M. Bryan and T. N. Morris, *Canning Trade J.* **3**, 252 (1933).

Read the samples against the artifical standard at 500 mμ. Subtract the reading for the solution at pH 3.4 from that for the same concentration of solution at pH 2.0. Convert this net reading to mg. of Congo red to give the same intensity. Multiply by 1.2 as the anthocyanin present and calculate back to the original sample.

Directly. As reagent mix 85 ml. of ethanol with 15 ml. of concentrated hydrochloric acid. Dissolve 0.001-0.05 mg. of residue from evaporation of a sample in 10 ml. of this reagent. If off-color brown, dilute with 50 ml. of water and extract with 10, 10 and 10 ml. of ether. Wash this extract with several portions of amyl alcohol, wash with water, and dilute to 25 ml. with the reagent before reading as above.

Bilirubin

Bilirubin is an orange-red crystalline compound which couples with diazotized sulfanilic acid to give a red color suitable for estimation.[19] Some oxidation also occurs as shown by similar color changes in acid solution without diazo reagent.[20] Accuracy to ± 2 per cent is obtainable up to 0.06 mg. per ml.[21] Beer's law does not hold over 0.016 mg. per ml.[22] Color develops for some time with the reagent so that the time of exposure should be standardized.[23] Alternative reagents are diazotized 4-nitro-4'-aminodiphenyl sulfone, 4,4'-diaminodiphenyl sulfone, 4,4'-diaminodiphenyl sulfide, and sulfamethazine.[24]

Bilirubin is present in both uncombined and combined forms in many samples, which leads to methods for estimation of the free and total amounts. Terminology for these is often direct and indirect bilirubin. Precipitation of protein causes low results. Therefore it is fortunate that in serum or plasma, at least, the presence of 40 per cent of methanol will catalytically permit determination of total bilirubin in a clear solution without deproteinizing.[25] Precipitation does not occur in 50 per cent methanol. The rose-mauve azobilirubin solution has a broad absorp-

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[19] A. A. H. van den Bergh and J. Snapper, *Deut. Arch. klin. Med.* **110**, 540-61 (1913).

[20] William Kerppola and Erkki Leikola, *Skand. Arch. Physiol.* **55**, 70-86 (1929).

[21] Arnold E. Osterberg, *J. Lab. Clin. Med.* **22**, 729-35 (1937).

[22] C. H. Gray and Joanna Whidborne, *Biochem. J.* **40**, 81-8 (1946); *Cf.* F. D. White and Dorothea Duncan, *Can. J. Med. Sci.*, **30**, 552-60 (1952).

[23] William D. Kelly, Jessica H. Lewis, and Charles S. Davidson, *J. Lab. Clin. Med.* **31**, 1045-9 (1946).

[24] Marcionilo Lins, *Arquív. biol.* Sao Paulo **35**, 78-81 (1951).

[25] Thomas B. Coolidge, *J. Biol. Chem.* **132**, 119-27 (1940).

ion band at 540 mμ. This band is unaffected by the presence of yellow serum pigments. Hemoglobin interferes but can be corrected by the use of a blank in adjusting the instrument. Feasible artificial standards include cobalt sulfate corrected by a green filter,[26] methyl red at pH 4.6-4.7,[27] and the Lovibond tintometer.[28] Sodium benzoate and urea promote coupling of the diazo reagent,[29] with the maximum at 530 mμ. In the absence of 1 ml. of serum in the reaction mixture the color is blue instead of red.[30]

The addition of caffeine sodium benzoate to diazo reagent lessens protein interference.[31] Theobromine can be substituted for caffeine and sodium salicylate [32] or sodium citrate [33] for sodium benzoate.

The blue product of oxidation of bilirubin with hydrogen peroxide is usually considered to be biliverdin although probably also containing bilicyanin.[34] Other oxidizing agents such as nitrite, nitrate, persulfate, and ferric chloride [35] have been used. If present, bilverdin will be reported as bilirubin by this method.[36] The color passes through shades from yellow to yellow-green, green, blue-green, and finally to blue, at which it is stable at room temperature for a considerable time. Sensitivity is increased by reading at 660 mμ. When introduced into the cup, readings must be made promptly [37] because light passing through the solution in the cup catalyzes the oxidation. The error of the method is not over ± 2 per cent at 0.0005 mg. of bilirubin per ml. of original sample.

Treatment of alkaline bilirubin with sulfuric acid, hydrochloric acid, or phosphoric acid in alcoholic solution gives first an olive color and then

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[26] Elizabeth Maclay and Arnold E. Osterberg, *Proc. Staff Meetings Mayo Clinic* **19**, 4-5 (1944); F. D. I. Pavlov, *Lab. Prakt.* (USSR) **15**, No. 6, 17-22 (1940).

[27] A. D. Haslewood and Earl J. King, *Biochem. J.* **31**, 920-3 (1937).

[28] M. R. Rudra, *Analyst* **71**, 326-7 (1946).

[29] W. N. Powell, *Am. J. Clin. Path., Tech. Sec.* **8**, 55-8 (1944); James J. Quigley, *Anal. Chem.* **24**, 1859-60 (1952).

[30] Elizabeth Maclay, *Am. J. Med. Technol.* **17**, 267-70 (1951).

[31] E. Enriques and R. Sivo, *Biochem. Z.* **169**, 152-60 (1926); *Rend. d. adunanze dell' accad. med. fis fiorentina, Sperimentale* **80**, 148-58 (1926); L. Jendrassik and A. Czike, *Deut. med. Wochschr.* **54**, 430 (1928); L. Jendrassik and M. Rebay Szabo, *Biochem. Z.* **294**, 293-9 (1937).

[32] Georg Lepehne, *J. Lab. Clin. Med.* **27**, 1447-52 (1942).

[33] F. Rappaport and F. Eichhorn, *Lancet* **1943**, I, 62-3.

[34] E. A. Peterman and Thomas B. Cooley, *J. Lab. Clin. Med.* **19**, 723-48 (1934).

[35] George E. Thoma and Doris M. Kitzberger, *Ibid.* **33**, 1189-92 (1948).

[36] Yosio Amada, *J. Biochem.* (Japan) **32**, 187-210 (1940).

[37] Helga Tait Malloy and Kenneth A. Evelyn, *J. Biol. Chem.* **119**, 481-90 (1937); *Ibid.* **122**, 597-603 (1937-38).

an intense green.[38] While the reaction occurs in the cold, it is accelerated at 50°. The usual constituents of bile or duodenal fluid do not interfere. The absorption curve differs from that for biliverdin. Results tend to be higher than by diazotized sulfanilic acid. The maximum absorption of the color developed it at 690 mμ.

Bilirubin can be extracted from an acidified sample and estimated by its color in chloroform.[39] Lipochrome can be co-extracted and interfere. A 2.5-mm. layer is appropriately read at 470 mμ. Combined bilirubin is sometimes determined by precipitation of protein with alcohol and the precipitate treated with methanol and dichloroacetic acid. The extract is matched against a standard made up of chrome alum, potassium bichromate, and nickel chloride.[40]

The amount of methylene blue required to convert the yellow of urine to green has been used as a rather unsatisfactory technic.[41] There is no chemical reaction of bilirubin with methylene blue.[42] The absorption by bilirubin in aqueous solution is at 435 mμ.[43]

Procedure—*By diazotized sulfanilic acid.* As reagent for development of color, freshly mix 10 ml. of 0.5 per cent sulfanilic acid in 1:70 hydrochloric acid with 0.1 ml. of 0.5 per cent sodium nitrite solution.

Urine. By addition of ascorbic acid the bilirubin does not change for 24 hours.[44] It is most satisfactory[45] to concentrate the bilirubin by sorption. Mix 10 ml. of urine with 5 ml. of a 5 per cent barium chloride solution. Shake and centrifuge. Discard the liquid and wash the precipitate twice with water. Add 1 ml. of diazo reagent to the precipitate

[38] W. Spiegelhoff and C. Gnann, *Klin. Wochschr.* 28, 719-20 (1950); *Z. ges. inn. Med. u. Grenzegebiete*, 7, 605-10 (1952).

[39] William Kerppola and Erkki Leikola, *Acta Med. Scan.* 78, 24-41, 277-80 (1932); R. J. Navarro and A. Tangco, *Acta Med. Philippina* 3, 21-32 (1941); Hector Ducci and Cecil J. Watson, *J. Lab. Clin. Med.* 30, 293-300 (1945).

[40] G. Ingvarsson, *Biochem. Z.* 294, 407-16 (1937).

[41] K. Franke, *Med. Klinik*, 27, 94-6 (1931); J. Seide and K. Zink. *Deut. med. Wochschr.* 57, 1744-5 (1931).

[42] Sydney S. Gellis and Joseph Stokes, Jr., *J. Am. Med. Assn.* 128, 782-3 (1945); Gordon D. Stokes, Earl E. Gambill, and Arnold E. Osterberg, *J. Lab. Clin. Med.* 31, 924-33 (1946).

[43] A. Dognon, *Compt. rend. soc. biol.* 129, 467-8 (1938).

[44] G. Barac, *Ibid.* 139, 412-14 (1945).

[45] Alvin G. Foord and Cecil F. Baisinger, *Am. J. Clin. Path.* 10, 238-44 (1940); Miguel C. Rubino and Nicolas Vojedovsky, *Anales asoc. quím. farm. Uruguay* 45, 25-7 (1942); F. Enríquez de Salamanca, F. Poggio Mesorana, and J. Oliver de la Gándara, *Trabajos inst. nacl. cienc. med.* (Madrid) 2, 7-13 (1943-4).

and shake. Add 1 ml. of 1:9 sulfuric acid and mix. Add 1 ml. of ethanol, mix, and centrifuge. Read after 30 minutes at 575 mμ [46] against a blank.

Alternatively,[47] mix 8 ml. of ethanol, 1 ml. of diazo reagent, and 1 ml. of urine. After 30 minutes, add 0.25 ml. of concentrated hydrochloric acid. Read at 575 mμ and 450 mμ. Calculate the corrected transmittance by equations which apply the corrections as follows.

$$\text{Per cent bilirubin} = 6.2[1.05 \times \text{density}_{575\text{m}\mu}) - (0.202 \times \text{density}_{450\text{m}\mu})]$$

Serum or plasma.[48] *Total bilirubin.* For development use 1 ml. of diazo reagent with 5 ml. of absolute methanol. To this add 4 ml. of 1:9 dilution of serum or plasma with water. Mix well and read after 30 minutes at 540 mμ against a blank in which the diazo reagent has been replaced with 1:70 hydrochloric acid.

Uncombined bilirubin. Follow the technic for total bilirubin but replace the methanol with water.

Hemoglobin present.[49] If serum or plasma contains hemoglobin, saturate a 5-ml. sample with carbon monoxide and add 0.5 ml. of 0.88 per cent ascorbic acid. Dilute to 50 ml. with acetone and filter. The hemoglobin will precipitate as the carbon monoxide compound.

Mix 20 ml. of filtrate with 2 ml. of the fresh diazo reagent. After 10 minutes add 1 ml. of concentrated hydrochloric acid and dilute to 25 ml. with acetone. Read at 570 mμ and subtract a blank to which no diazo reagent was added.

Feces.[50] The feces must be acid to avoid oxidation. If alkaline add acetic acid. By extraction with chloroform, in which biliverdin is insoluble, the reaction is specific. Pulverize a 5-gram sample with a few grams of anhydrous sodium sulfate and grind with 5 ml. of chloroform. Shake mechanically for 3-4 hours with 10 ml. of chloroform and filter.

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[46] Cf. Charles Sheard, *Proc. Staff Meetings Mayo Clinic* **15**, 421-4 (1940); W. H. Goodson and Charles Sheard, *J. Lab. Clin. Med.* **26**, 423-33 (1940-1).

[47] Walter R. D. Golden and John G. Snavely, *Ibid.* **33**, 890-903 (1948).

[48] Helga Tait Malloy and Kenneth A. Evelyn, *J. Biol. Chem.* **119**, 481-90 (1937); Roberto Battistessa, *Rev. faculdad cienc. quim.* **18**, 155-68 (1943); Bernardo Supelvada and Arnold E. Osterberg, *J. Lab. Clin. Med.* **28**, 1359-68 (1943); Cf. W. J. H. Bungenberg de Jong, *Klin. Wochschas* **20**, 885-6 (1942); William Kerppola, *Acta Med. Scand.* **112**, 291-301 (1942).

[49] Max Engel, *Z. physiol. Chem.* **259**, 75-82 (1939).

[50] Torben K. With, *Z. physiol. Chem.* **275**, 166-75 (1942); Martin Madel, *Z. ges. inn. Med.* **2**, 659-65 (1947); Wilhelm Kunzer, Judith Zanner, and Hans Zeisel, *Klin. Wochschr.* **28**, 681-3 (1950).

Wash the residue with chloroform until a 25-ml. filtrate is obtained and then concentrate a 10-ml. aliquot to 2 ml. on a steam bath. Cool and add 5 ml. of ethanol and 1 ml. of freshly prepared diazo reagent. After 15 minutes add a few crystals of anhydrous sodium sulfate, shake, filter, and dilute to 10 ml. with ethanol. Acidify with a drop or two of concentrated hydrochloric acid and read the blue color at 570 mμ against a blank from which the reagent was omitted.

By caffeine sodium benzoate and diazo reagent.[51] Dissolve 30 grams of sodium benzoate and 20 grams of caffeine in 70 ml. of water. Prepare also a three-quarter saturated solution of sodium acetate. Add 1 ml. of the sodium benzoate reagent and 1 ml. of the sodium acetate reagent to 1 ml. of sample. Add 1.5 ml. of water and 0.5 ml. of the diazo reagent and read at 530 mμ against a reagent blank.

By hydrogen peroxide. As reagent, mix 0.4 ml. of 30 per cent hydrogen peroxide and 2 ml. of concentrated hydrochloric acid in 100 ml. of ethanol.

Bile. Add 9.5 ml. of ethanol and 10 ml. of reagent to 0.5 ml. of fresh bile. Mix and filter after 1 hour. Read with a 660-mμ filter against a reagent blank.

Feces. Stir a 24-hour specimen mechanically with 500 ml. of water for 3-5 hours to give a homogeneous dispersion. An extremely fine suspension of particles is essential for good results. Mix a 10-ml. aliquot of the dispersed sample with 15 ml. of ethanol and 25 ml. of reagent and stir vigorously. Centrifuge after 1 hour and pour off the supernatant liquid. Add 25 ml. of ethanol and 25 ml. of oxidizing reagent to the precipitate. Stir vigorously, allow to stand for 1 hour, and filter. Read along with the supernatant liquid of the first extraction with a 660-mμ filter against ethanol as a blank.

Urine.[52] Mix 2 ml. of urine, 2 ml. of 20 per cent sulfosalicylic acid solution, and 5 drops of reagent. Shake and allow to stand for an hour. If bilirubin is absent, a red color will form. If it is present, a green color will develop within a few minutes which varies in intensity with the amount present. Read with a 660-mμ filter against a blank from which hydrogen peroxide was omitted.

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[51] L. Jendrassik and R. A. Cleghorn, *Biochem. Z.* **289**, 1-14 (1936); M. R. Cortax, A. Lopez Garcia, and J. F. Zelasco, *Rev. soc. Argentina. biol.* **16**, 257-70 (1940); Torben K. With, *Acta Med. Scand.* **115**, 542-52 (1943); *Acta Phys. Scand.* **10**, 181-91 (1945).

[52] Bela von Purjesz, *Med. Klin.* **33**, 1271 (1937).

By ferric chloride. As reagent mix 5 grams of trichloroacetic acid, 20 ml. of water, and 2 ml. of 35 per cent solution of ferric chloride hexahydrate.

Serum or urine. Mix 10 ml. of sample with 10 ml. of reagent and read against a reagent blank.

By strong acid. Bile. Mix 1 ml. of bile and 19 ml. of a 1:6 mixture of 1:2.6 sulfuric acid and ethanol. Incubate at 50° for 20 minutes and read at 620 mμ.

Extraction with chloroform. Serum or plasma. Add 2 ml. of chloroform to 1 ml. of serum or plasma and shake vigorously. If bilirubin is absent the solution remains colorless. If it is present the chloroform layer which separates after a few minutes is yellow or green-yellow in color. Transfer the chloroform layer and re-extract the sample until the separated chloroform layer is colorless. Compare the color of the combined chloroform extracts against 1:6000 potassium bichromate which has a color equivalence of 0.329 mg. of bilirubin per 100 ml.

Urobilin and Urobilinogen

These decomposition products of bilirubin are coalesced in their determination because light and oxygen form urobilin from urobilinogen. Similarly the addition of a reducing agent such as ferrous salt [53] converts urobilin to urobilinogen. This consists of mesobilene and tetrahydromesobilene [54] of which the latter predominates. Oxidized, it forms tetrahydromesobilene. There is no reaction of urobilin with *p*-dimethylaminobenzaldehyde, but, when converted to urobilinogen, it is determined by the red to purple color.[55] There is some interference from the yellow

[53] A. J. L. Terwen, *Nederland, Tijdschr. Geneeskunde* **69**, **I**, 2492-2507 (1925); A. Lichtenstein and A. J. L. Terwen, *Deut. Arch. klin. Med.* **148**, 72-101 (1925); L. Heilmeyer, *Z. ges. exptl. med.* **76**, 220-35 (1931); L. Heilmeyer and W. Krebs, *Biochem. Z.* **231**, 393-8 (1931); Cecil J. Watson, *Am. J. Clin. Path.* **6**, 458-75 (1936); H. N. Naumann, *Biochem. J.* **30**, 347-51 (1936); E. H. Bensley, *J. Lab. Clin. Med.* **21**, 1195-7 (1936); Laurence Farmer, *Ibid*, 22, 1277-8 (1937); Robert Sparkman, *Arch. Internal Med.* **63**, 858-66 (1939); F. I. Simon, *Schweiz. med. Wochschr.* **71**, 141-2 (1941); F. Fischler, *Munch. med. Wochschr.* **89**, 336-7 (1942); S. Schwartz, V. Sborov, and C. J. Watson, *Am. J. Clin. Path.* **14**, 598-604 (1944); C. J. Watson, S. Schwartz, V. Sborov, and Elizabeth Bertie, *Ibid.* **14**, 605-15 (1944); C. J. Watson and Violet Hawkinson, *Ibid.* **17**, 108-16 (1947).

[54] R. Lemberg, W. H. Lockwood, and R. A. Wyndham, *Australian J. Exptl. Biol. Med. Sci.* **16**, 169-80 (1938).

[55] Ehrlich, *Gentr. klin. Med.* **8**, 593-4 (1887); Hugh G. Brereton and S. P. Lucia, *Am. J. Clin. Path.* **18**, 887-90 (1948).

color of the reagent as well as from that of samples such as urine. With urea or acetone it gives a greenish yellow. Nitrite inhibits the reaction. A green color indicates biliverdin from simultaneous presence of nitrite and bilirubin.

Porphobilinogen gives a similar result with different absorption of the end product which is easily extractable with organic solvents. By reversing the order of addition of the reagents, the background color is corrected.[56] Oxidation with nitric acid in hydrochloric acid is also applicable.[57]

Procedure—*By p-dimethylaminobenzaldehyde.* As reagent dissolve 5 grams of *p*-dimethylaminobenzaldehyde in 100 ml. of 1:1 hydrochloric acid and dilute to 150 ml.

Urine. Urobilinogen. To 1.25 ml. add 1.25 ml. of reagent. Mix and add 2.5 ml. of saturated aqueous sodium acetate. As a blank reverse the order of addition of the buffer and reagent to avoid color development. Read the sample against water and subtract the blank.

Urobilin. Mix 15 ml. of urine with 1 ml. of saturated ferrous ammonium sulfate solution. Stir at room temperature in the dark and gradually add 1 ml. of 10 per cent sodium hydroxide solution with stirring. Protect from exposure to the air for a time and filter. Measure out 11.33 ml. of the filtrate, equivalent to 10 ml. of the original sample. Add 20 per cent tartaric acid solution in sufficient quantity to make distinctly acid. Extract with 40 ml. of ether and separate the ether extract. Wash this extract with four small portions of water.

Mix 30 ml. of the ether extract of urobilinogen, 3 ml. of a saturated solution of *p*-dimethylaminobenzaldehyde in ether, and 10 drops of concentrated hydrochloric acid. Shake vigorously for 90 seconds to condense the urobilinogen with the reagent to give a purple dye. Shake the ether solution with 3 ml. of saturated sodium acetate solution to remove excess of hydrochloric acid and read. Subtract the urobilinogen as determined separately from this to get the urobilin.

Feces. Urobilinogen. Mix a 5-gram sample, 140 ml. of water, and 5 grams of ferrous sulfate and agitate mechanically for 10 minutes. Add 40 ml. of 10 per cent sodium hydroxide solution and stir. Allow to stand overnight and filter. Mix 5 ml. of filtrate, 4.5 ml. of water, 2.5 ml.

[56] Lawrence E. Young, R. Wendell Davis, and Jane Hogestyn, *Ibid.* 34, 287-91 (1949).

[57] Karl Singer and Rosa Kubin, *Ibid.* 28, 1042-9 (1943).

of 1:1 hydrochloric acid, and 1 ml. of reagent. As a blank, mix 5 ml. of filtrate, 1 ml. of 2.6 per cent sodium hypochlorite solution, 2 ml. of 1:1 hydrochloric acid, and 1 ml. of reagent. Read the sample against the blank.

Urobilin. Mix 1.5 grams of fresh feces with 13.5 ml. of water by shaking. Complete as for urobilin in urine, starting at addition of ". . . 1 ml. of saturated ferrous ammonium sulfate solution.

Coproporphyrin

This can be read by the natural red color.[58] One standard is commercial hematoporphyrin hydrochloride, the results being multiplied by 0.7.

Procedure—To 500 ml. of urine add 50 ml. of 20 per cent sodium hydroxide solution. Add a little dicalcium phosphate. Centrifuge and wash the precipitate with 50 ml. of water. Dissolve the precipitate in 10 ml. of acetic acid and add 10 ml. of water. Extract the coproporphyrin with 25, 15, and 10 ml. of ether. Extract from the ether with 10 ml. of 1:3 hydrochloric acid. Drive off the ether, cool, dilute to 10 ml., and read at 550 mμ.

Alternatively acidify 15 ml. of urine with 1 ml. of glacial acetic acid and extract with 30 ml. of ether. Wash the separated ether with water and extract with five 1-ml. portions of 1:120 hydrochloric acid. Filter the extracts and read at 380, 401, and 430 mμ. Calculate from

$$D_{\mathrm{Cor}} = [2D_{401} - (D_{380} + D_{430})]/1.833$$

Uroporphyrin

Uroporphyrin is precipitated on calcium hydroxide, dissolved in acid and read.[59]

Procedure—Mix 1 ml. of urine with 1 ml. of buffer for pH 5-5.5 and add 2 drops of 5 per cent disodium phosphate solution. Add 2 ml. of 3 per cent calcium chloride solution and 2 ml. of 4 per cent sodium

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[58] Konrad Dobriner and C. P. Rhoads, *New Engl. J. Med.* **219**, 1027-9 (1938); Atusi Sato, *Klin. Wochschr.* **17**, 1108-11 (1938); Paul L. Eging and Theodore Cornbleet, *J. Investigative Dermatol.* **5**, 127-33 (1941); R. Askevold, *Scand. J. Clin. and Lab. Invest.* **3**, 318-19 (1951).

[59] R. Askevold, *Scand. J. Clin. and Lab. Invest.* **3**, 318-19 (1951).

hydroxide solution. Similarly mix 1 ml. of urine with 1 ml. of buffer and heat in boiling water for 30 minutes without evaporation. Cool and treat as for first sample. Centrifuge, each decant, and wash the precipitate with 0.4 per cent sodium hydroxide solution and with water. The upper layer should show no fluorescence up to this point; if it does the calcium chloride is inadequate. Dissolve the precipitates in 10 ml. of 1:24 hydrochloric acid and read at 380, 405, and 430 mμ against 1:24 hydrochloric acid. Subtract the blank and calculate

$$D_{Cor} = [2D_{405} - (D_{380} - D_{430})]/1.844$$

Protoporphyrin

Protoporphyrin is extracted from a mixture of red cells with 3:1 ethyl acetate and acetic acid. The extraction medium is washed and extracted with 1:10 hydrochloric acid. After transfer to ether and back to acid, reading is by the intensity of red fluorescence or the color present.[60]

Procedure—*Corpuscles.* Wash 5 ml. of red blood cells twice with physiological saline solution and then homogenize with 50 ml. of a 3:1 mixture of ethyl acetate and glacial acetic acid. Shake for 1-2 minutes and decant the supernatant liquid through filter paper. Wash the residue three times with 15-ml. portions of the acetate-acid mixture. Wash the filtrate three times with water. Extract the combined water washings with 10 ml. of ethyl acetate to remove any protoporphyrin present and add to the main ethyl acetate fraction. Allow the aqueous layer to separate completely and remove. Extract the ethyl acetate fraction with 2-ml. portions of 1:10 hydrochloric acid until no further fluorescence is observed when using a Corning 587 filter. Buffer the combined hydrochloric acid extracts by addition of solid sodium acetate until it no longer reacts acid to Congo red paper. Then add 1 ml. of glacial acetic acid to reduce the tendency to form emulsions during the subsequent extraction.

[60] A. A. Hymans van den Bergh and A. J. Hyman, *Deutsch. med. Woch.* **54**, 1492-4 (1928); A. A. Hymans van den Bergh and W. Grotepass, *Klin. Woch.* **12**, 586-9 (1933); K. Lageder, *Ibid.* **15**, 296-8 (1936); Enrico Vigliani and Claudio Angeleri, *Ibid.* **15**, 700-1 (1936); *Rass. med. applicata lavoro ind.* **7**, 91-102 (1936); O. Schumm and G. Knop, *Z. ges. exp. med.* **106**, 252-95 (1939); K. Seggel, *Ergebn. inn. Med. u. Kinderh.* **58**, 582 (1940); Moises Grinstein and Cecil James Watson, *J. Biol. Chem.* **147**, 675-84 (1943).

Extract the solution 3 times with equal volumes of ethyl ether. Wash the combined ether extracts with a few ml. of 1 per cent sodium carbonate solution, then wash twice with water. Next, extract the ether 3 or 4 times with 1.5 ml. portions of 1:20 hydrochloric acid until fluorescence is no longer observed. Combine the acid extracts and dilute to 10 ml. with 1:20 hydrochloric acid. Mix well and read against a reagent blank at 400 mμ. Alternatively read the fluorescence.

Porphobilinogen

The porphyrins of urine are converted by acid to uroporphyrin and porphobilin, collected on calcium phosphate, and dissolved for reading.[61] This can also be developed with *p*-dimethylaminobenzaldehyde.[62]

Procedure—*By direct reading. Urine.* Mix 50 volumes of sample with 1 volume of concentrated hydrochloric acid. Allow to stand for 24 hours or heat for 10 minutes in boiling water. For urine low in phosphate add more as trisodium phosphate solution. Add 1 ml. of 3 per cent calcium chloride solution and 2 ml. of 4 per cent sodium hydroxide solution. The porphyrins are with the calcium phosphate precipitate. Wash the precipitate with 0.4 per cent sodium hydroxide solution and then with water. Dissolve the precipitate in 10 ml. of 1:25 hydrochloric acid and filter. Read at 430, 380, and 405 mμ. Then the uroporphyrin is $P_{405} = [2D_{405} - (D_{430} + D_{380})]/1.844$. Calculate on a basis $E^{1\%}_{1\,\text{cm.}} = 5000$.

By p-*dimethylaminobenzaldehyde.* Make 5 ml. of urine distinctly acid with acetic acid and extract with 2 ml. and 2 ml. of ether. Read the extracted urine at 550 mμ as the blank. Add 1 ml. of 3.33 per cent solution of *p*-dimethylaminobenzaldehyde in 1:2 hydrochloric acid and read again at 550 mμ.

Hemovanidin

Hemovanidin is a term which has been assigned to a pigment from hemolyzed blood corpuscles of mammalian origin.[63]

Procedure—Read the absorption of hemolyzed blood corpuscles at 297.5 mμ.

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[61] C. Rimington and H. D. Barnes, *Scand. J. Clin. and Lab. Invest.* 1, 2-11 (1949).
[62] Bo Vahlquist, *Z. physiol. Chem.* **259**, 213-21 (1939).
[63] L. Califano and P. Caselli, *Pubbl. staz. zool. Napoli* **22**, 138-45 (1950).

Cytochrome C

Cytochrome is a pigment related to hemoglobin but differing in that the protein is not linked by partial valences alone to iron. For determination the cytochrome C is isolated free of interfering pigments, reduced with sodium thiosulfate, and determined as ferrocytochrome C by direct reading.[64] The method is applicable to samples weighing 0.20-20 grams and having a cytochrome C concentration of 0.001-0.3 mg. per gram.

Another method[65] is based on the facts that (a) reduced cytochrome C has a greater absorption than oxidized cytochrome C at a wave length of 550 mμ, (b) the total absorption of a solution containing several solutes is equal to the sum of their individual absorptions when there is no interaction, and (c) cytochrome C can be oxidized and reduced enzymatically without altering the absorption of the other components of the test solution. For this cytochrome C is reduced by an enzyme containing succinic dehydrogenase and cytochrome oxidase.[66] A small amount of cyanide, followed by excess succinate, gives cytochrome C in the reduced form.

Since cytochrome is an iron derivative, it is measured through that constituent.[67] For this the complex is decomposed by sodium hydroxide and hydrogen peroxide, the hydrogen peroxide removed, the pH adjusted to 4.2-4.3 by hydrochloric acid and ammonium acetate, the iron reduced with ascorbic acid, and determined with *o*-phenanthroline against a ferrous iron standard. When hydrolyzed the porphyrin part of the cytochrome C molecule gives a maximum absorption around 420 mμ.[68]

Sample—*Tissue*. Mince the tissue finely, mix thoroughly, rinse with Ringer's solution, and drain. Grind a weighed amount in a mortar with powdered Pyrex glass. Then grind successively with the following per gram: 0.25 ml. of water, 1.25 ml. of 1:35 sulfuric acid, and 0.5 ml. of 1:7.5 ammonium hydroxide. Allow the mixture of extracts and ground tissue to stand with occasional stirring for 15 minutes. Centrifuge,

[64] Akuzi Huzita, Tozyu Hata, Iosama Numata, and Masanobu Azisaka, *Biochem. Z.* **301**, 376-90 (1939); Otto Rosenthal and David L. Drabkin, *J. Biol. Chem.* **149**, 437-50 (1943).

[65] V. R. Potter and K. P. duBois, *Ibid.* **142**, 417-26 (1942); A. Prader and A. Gonella, *Experientia* **3**, 462-4 (1947).

[66] V. R. Potter, *J. Biol. Chem.* **137**, 13-20 (1941).

[67] David L. Drabkin, *J. Optical Soc. Am.* **31**, 70-2 (1941).

[68] H. Theorell, *Biochem. Z.* **285**, 207-19 (1936).

remove the turbid supernatant liquid, and record its volume. It represents 60-70 per cent of the volume of the original mixture. To the supernatant liquid add an equal volume of 50 per cent ammonium sulfate solution. Stopper and place in a 56° bath for 15 minutes. The cytochrome C and some spectroscopically-inert proteins remain completely soluble, whereas hemoglobin and other proteins start to flocculate immediately which continues through the period of heating. Let the mixture stand overnight and filter.

To the whole filtrate or a suitable aliquot, add 1 ml. of 90 per cent trichloroacetic acid per ml. of filtrate, mix, and refrigerate for 30 minutes. Flocculation should occur within 30 minutes. If the mother liquor remains turbid, add 0.5 gram of ammonium sulfate per 10 ml. of initial filtrate and repeat this addition until precipitation of the cytochrome C-containing material is complete. When precipitation is complete, rinse the stirrer with saturated ammonium sulfate solution and centrifuge for 30 minutes. Remove the supernatant liquid and check for turbidity. Drain the tube containing the precipitate by inversion on filter paper and then dissolve the precipitate by adding 4 per cent sodium hydroxide solution dropwise, usually 0.25 ml. A small amount of material will remain. This is probably denatured protein. Dilute to 1 ml. with water or with sufficient 1:10 hydrochloric acid to make the final hydroxyl ion concentration about 0.2 *M*. Centrifuge and remove insoluble material.

Procedure—Read at 630 mμ.

Urochrome

Urochrome is extracted and read directly.[69]

Procedure—*Urine.* Saturate 100 ml. with ammonium sulfate at room temperature. Extract the urochrome with 30 ml. of ethanol. Filter and read at 480, 500, and 530 mμ. Average the readings at the three wave lengths for comparison.

Alternatively, mix 20 ml. of fresh urine with 4 ml. of acetic acid and 30 ml. of absolute ethanol. Extract with 20 ml. of chloroform. Wash the chloroform with 30 ml. and 15 ml. of water. Add the washings to the previous aqueous layers. The chloroform contains the urobilin and uroerythrin. Save for separate determination. Dilute the aqueous layer to 100 ml. and read at 530 mμ. This is the urochrome.

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[69] G. G. Yaure, *Compt. rend. acad. sci.* (USSR) **28**, 663-5 (1940); Atusi Sato, *Klin. Wochschr.* **17**, 1108-11 (1938).

Uroerythrin

Uroerythrin is extracted from urine for reading.[70]

Procedure—*Urine.* If the uric acid content is low, add 1 ml. of 1 per cent sodium urate to a 20-ml. sample. Saturate the sample with ammonium chloride. Centrifuge. Wash the precipitate with 3 ml. of water and centrifuge. Dissolve in 1 ml. of acetic acid and 5 ml. of absolute ethanol. Add 5 ml. of water and shake with 10 ml. of chloroform. Wash the chloroform extract with 10 ml. of water and separate. Dilute to 10 ml. with absolute ethanol and read at 530 mμ.

Oxychrome

The pigments in urine are read as oxychrome.[71]

Procedure—Acidify 10 ml. of urine with acetic acid. Add bleaching powder and after 30 minutes read at 530 mμ.

Noregonolidin Acetate, Egonol

Egonol gives a scarlet color on oxidation with hydrogen peroxide.[72] Phytosterol or octadecyl alcohol in excess of the egonol does not interfere.

Procedure—Dissolve the sample in glacial acetic acid at about 1 mg. per ml. To 10 ml. add 1 ml. of 35 per cent hydrogen peroxide. Place in a water bath at 80° for 15 minutes and cool. Read within 10 minutes.

Lactucin, Lactucopicrin

After reaction with potassium cyanide in 50 per cent methanol, a color is developed with alkali.[73]

Procedure—Mix 5 ml. of a methanol solution and 5 ml. of 7 per cent aqueous sodium cyanide solution. After 10 minutes add 10 ml. of 2 per cent sodium hydroxide in methanol. After 15 minutes at room temperature, read at 280-400 mμ.

[70] Atusi Sato, *Klin. Wochschr.* **17**, 1108-11 (1938).
[71] *loc. cit.*
[72] Hiromichi Matsubara, *Botyu Kagaku* **16**, 99-102 (1951).
[73] Gerhard Schenck and Fritz Wendt, *Mikrochemie ver. Mikrochim. Acta* **38**, 554-65 (1951).

Peptone

The biuret reaction of albumoses and peptones with copper sulfate can be applied to quantitative estimation of peptones.[74] Proteins must be removed by boiling at a suitable pH. Amino acids do not interfere. The method was developed for application to nutrient media for bacteria. It is accurate to about 7.5 mg. for 18-90 mg. and most accurate at 25-40 mg.

Procedure—To 10 ml. of sample containing 0.5-1 per cent of peptone add 40 ml. of water and mix. Add 5 ml. of 4 per cent sodium hydroxide solution and mix. Add 2 ml. of 2.5 per cent copper sulfate pentahydrate solution and mix. Dilute to 70 ml., mix, and let stand for 5 minutes. Read against a reagent blank.

Pitch

Pitch in paper pulp predicting trouble on the screens, is determined by the resinous and fatty acids.[75]

Procedure—Shake a 0.5-gram sample of air-dried pulp with 6 ml. of acetic anhydride and 2 ml. of carbon tetrachloride. Filter and add 0.2 ml. of concentrated sulfuric acid. Shake for 2-3 minutes and compare with standards.

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[74] E. Bugareff and E. Sergejewa, *Biochem. Z.* **255**, 88-91 (1932).

[75] M. N. Beletsckaya, *Tsentral. Nauch-Isoledovatel. Inst. Bumazh. Prom. Materialy* **1937**, No. 25, 127-36.

CHAPTER 16

COLOR OF LIQUIDS [1]

The methods of colorimetric analysis primarily consist of development of a more or less stable color in solution and reading it at some optimum condition. On the other hand, there are a multitude of uses of the present-day instruments for reading of colors not specifically developed for the purpose. Inclusion of all is out of the question. A few typical cases of both aqueous and nonaqueous liquids are shown.

Water

The "apparent color" of water is the color of the original unfiltered sample and includes the color of any substance in suspension. The "true color" is that due only to substances in solution. Color is measured by comparison with platinum-cobalt solutions.[2] The ratio of platinum to cobalt may be varied in special cases.

Standards—Dissolve 1.245 grams of potassium chloroplatinate, containing 0.5 gram of platinum, and 1 gram of crystallized cobalt chloride, containing about 0.25 gram of cobalt, in water with 100 ml. of concentrated hydrochloric acid and dilute to 1 liter with water. To substitute chloroplatinic acid for the chloroplatinate, dissolve 0.5 gram of metallic platinum in *aqua regia*. Remove nitric acid by repeated evaporation to dryness after adding an excess of concentrated hydrochloric acid. Dissolve this product in water and add the cobalt chloride as above. The solution so prepared has a color of 500.

Prepare a series of standards by measuring 0.5, 1.0, 1.5, 2.0 ml., etc., up to 7 ml., of the standard color into 50-ml. Nessler tubes. Dilute each with water to 50 ml. Each ml. of standard color diluted to 50 ml. gives a color of 10, the series of standards thus being 5, 10, 15, 20, etc., to 70. Seal the standards to protect them from dust and evaporation when not in use.

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[1] See Volume III, Chapter 1, for details of organization, condensation, etc.

[2] Allen Hazen, *Am. Chem. J.* 12, 427-8 (1892); "Standard Methods for the Examination of Water and Sewage" 9th ed., p. 4 American Public Health Association New York N. Y., (1946).

Procedure—*True Color.* Filter the water through filter paper or preferably through a Berkefeld filter. A Pasteur filter must not be used since it has a marked decolorizing action. Fill a standard Nessler tube to the 50-ml. mark, which will be the same level as the standards. Compare by looking downward through the tubes to a white or mirrored surface which reflects light upward through the solutions. If a sample has a color value greater than 70, dilute with water accurately. Record colors 1-50 to the nearest unit, 51-100 to 5 units, 101-250 to 10 units, and 251-500 to 20 units.

Apparent color. Examine the unfiltered water in the same way.

Field Work—Make comparison with glass disks at the end of metallic tubes, calibrated to correspond with colors on the platinum scale.

Flavoring Extracts

The color of flavoring extracts is typical of readings in terms of Lovibond glasses, not yet taken over into the photometric field.[3]

Procedure—*Color value.* Dilute 2 ml. to 50 ml. with 50 per cent ethanol. Read in terms of red and yellow Lovibond glasses in a 1-inch cell and multiply by 25 for the value.

Residual color after precipitation with lead acetate. Dilute 50 ml. to 80 ml. with water and evaporate back to 50 ml. on a water bath at not over 70°. Repeat. Add 25 ml. of 8 per cent neutral lead acetate solution and dilute to 100 ml. with water. Shake and let stand overnight at 37-40°. Filter and read in a 1-inch cell against Lovibond glasses. Multiply by a factor to convert to the original extract.

Color insoluble in amyl alcohol. Evaporate a 25-ml. sample to dryness on a steam bath. Take up in 26.3 ml. of alcohol and water to make 50 ml. Extract 25 ml. with fresh reagent containing 100 ml. of amyl alcohol to 3 ml. of 85 per cent phosphoric acid and 3 ml. of water. Avoid so shaking as to form emulsions and draw off the lower layer. Dilute to 25 ml. with 50 per cent ethanol and compare by balancing against the untreated 25 ml. from the original dried sample. Report as per cent of color insoluble in amyl alcohol.

[3] Official and Tentative Methods of the Association of Official Agricultural Chemists, 7th Ed. p. 308, Association of Official Agricultural Chemist, Washington, D. C. (1950).

Beer and Wort

Beer is read in absolute terms.[4]

Sample—*Beer.* Partially degas by swirling.

Procedure—*Spectrophotometric.* For this a spectrophotometer with a wave band width of 1 mμ or less is essential. Read at 430 mμ and 700 mμ in a 0.5-inch cell as rapidly as possible. If the value for optical density at 430 mμ $\times$0.039 exceeds that at 700 mμ, the beer is free from turbidity. Then the beer color intensity is 10 times the optical density at 430 mμ. Otherwise report the beer color as 10 (optical density$_{430}$ − [optical density$_{700}$ − 0.03 optical density$_{430}$]).

Photometric. Read at 420-450 mμ.

Indigo

When dispersed in water in colloidal form by precipitation of the sulfate from concentrated sulfuric acid, indigo is read subject to the error introduced by reproducibility of the particle size.[5] Alternatively the dye is extracted from the fabric as the leuco base and reoxidized. A solution of mixed indigo and isatin in aniline will give both at 610 mμ and 470 mμ respectively.[6] At 0.002-0.02 mg. per ml. the indigo can be read within 1 per cent. An alternative standard is Sirius blue for visual comparison.

Samples—*Fabric.* Treat a sample exactly 5 cm. square with 20 ml. of concentrated sulfuric acid for 45 minutes. A dark green solution is produced with the indigo present as the sulfate. Tip this into 750 ml. of cold water and rinse in the balance with water. Dilute the colloidal dispersion of indigo to 1 liter for reading.

Pastes. To prepare a solution of sodium protoalbinate, combine 16 grams of sodium hydroxide with about 525 ml. of water and 100 grams of ground egg albumin. Shake and heat on a water bath for an hour to dissolve. Filter if turbid and dilute to 500 ml.

Combine a sample estimated to contain 0.25 gram of indigo, 100 ml.

[4] *J. Assocn. of Official Agr. Chem.* **34**, 61-3 (1951).

[5] J. Lotichius, *J. Soc. Dyers Colourists* **55**, 87-8 (1939); *Ibid.* **56**, 433-5 (1940). Cf. Petrov, *Legkanya Prom.* **15**, No. 7, 86 (1936); J. Lotichius and J. Kooyman, *J. Soc. Dyers Colourists* **67**, 435-6 (1951).

[6] Maurice Martin and Raymond Raillère, *Am. chim. anal.* **28**, 52-3 (1946).

of water, 10 ml. of 40 per cent sodium hydroxide solution, 7 ml. of sodium protoalbinate solution, and about 2 grams of sodium hydrosulfite. Stopper and let stand overnight at under 30°. Add 3 per cent hydrogen peroxide until further additions do not intensify the color and dilute to 250 ml. Dilute 5 ml. to 1 liter for reading.

As a rapid method mix 2 mg. of paste with 10 ml. of concentrated sulfuric acid. After at least 2 hours at room temperature with occasional stirring, pour into water and dilute to 1 liter. Dilute a portion with an equal volume of water and read.

Indigo vat. Mix 25 ml. of sodium protoalbinate solution by introduction of 25 ml. of indigo vat below the surface. Add 5 ml. of 3 per cent hydrogen peroxide and mix. Dilute to 100 ml. and dilute an aliquot according to strength.

Isatin-indigo mixtures. Reflux 25 mg. with 50 ml. of aniline until dissolved and filter rapidly from extraneous material.

Procedure—Read the solutions at 610 mμ and compare with a calibration curve similarly prepared. If isatin is also present read it at 470 mμ.

Color of Fats and Oils

The following spectrophotometric method has been adopted by the American Oil Chemists' Society.[7]

Definition: This method denotes the color of an oil by determining the optical density at a specified wave length of light, using a spectrophotometer.

Scope: Applicable to fats and oils.

A. Apparatus

1. *Spectrophotometer*—A spectrophotometer capable of adjustment to give the following readings after setting the zero point and after adjusting the 100 per cent transmittance point (0 density) against carbon tetrachloride, in a cuvet having the specifications outlined in (2) below.

400 millimicrons	Less than 4 per cent transmittance
470 millimicrons	38.2 ±2
510 millimicrons	73.4 ±2
525 millimicrons	69.4 ±2
550 millimicrons	54.4 ±2
700 millimicrons	Less than 2 per cent

[7] J. Worthington Agee, *J. Am. Oil Chemists Society* 26, 312-16 (1949).

2. Matched glass cylindrical cuvets, approximately 21.8 mm. inside diameter; outside diameter approximately 24.5 mm.—All cuvets should check carbon tetrachloride and the nickel sulfate solution at 525 mμ within ±0.6 per cent transmittance. The cuvets should be kept clean and free from scratches.

3. Standardizing Nickel Sulfate Solution—Dissolve 200 grams of nickel sulfate hexahydrate $NiSO_4 \cdot 6H_2O$, in water. Add 10 ml. of concentrated hydrochloric acid. Dilute to exactly 1000 ml. The temperature of the solution should be between 25 and 30° C. The density of the solution at 25° C. should be 1.115 and nickel content must fall between 3.95 and 4.00 per cent nickel by analysis.

4. Filter paper—Fine porosity such as E & D No. 192, Whatman No. 12, Reeve-Angel No. 871, or S & S No. 596.

B. Reagents

1. Carbon tetrachloride—Redistilled if the transmittance differs from distilled water by 0.5 per cent at 400 mμ.

C. Procedure

1. The sample must be absolutely clear. If not, filter through a specified paper at a temperature of at least 10° C. above the melting point of the fat. The sample should not be held melted longer than necessary since darkening may occur.

2. Turn on the spectrophotometer and allow at least a 20-minute warm-up period before standardizing or making any measurement.

3. Set the wave-length scale to the desired wave length.

4. Recheck the zero reading of the instrument and, with a cuvet filled with carbon tetrachloride in the instrument, set the 100 per cent transmission point exactly.

5. Fill a cuvet with a standardizing nickel sulfate solution and read the transmittance at 400 mμ. Repeat 3 and 4 at 470, 510, 525, 550, and 700 millimicrons. The readings must fall within the limits prescribed, or the instrument should be adjusted to give the correct response.

6. Fill a cuvet with the sample, using a sufficient amount of oil to insure a full column in the light beam.

7. Place the filled tube in the instrument and read the optical density to the nearest 0.001 at 525 millimicrons.

Reporting:

1. Report the optical density multiplied by 1000.

$$(\text{Red color} = \text{density} \times \text{factor})$$

Factors: up to 0.085 density

$$\text{red} = \text{density} \times 42$$

above 0.085 density

$$\text{red} = 23.4\ \text{density} + 1.52$$

Special instrument scales for reading red colors directly may be used.

AUTHOR INDEX

SUBJECT INDEX